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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

The Journal is available world-wide.

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Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

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In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

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2. The CEE sends the article-identifying information having been removed, to three reviewers who are specialists in the subject matter represented by the article. The CEE requests them to complete the review in three weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the field.

3. The CEE, in consultation with the Editor-in-Chief (EiC), examines the reviews and decides whether to reject the manuscript, invites the author(s) to revise and resubmit the manuscript. The CEE may seek additional reviews. Final acceptance or rejection rests with the CEE and EiC, who reserve the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The CEE sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the CEE in consultation with the EiC and EBMs examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear online in approximately three months.

   The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the article appears in the pages of the Journal and is posted online.

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

Pertanika is over 40 years old; this accumulated knowledge has resulted in the journals being abstracted and indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index [ESCI (Web of Science)], BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite and ISC.

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Contents

Foreword

Welcome to the Third Issue of 2019 for the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 19 articles; 2 are review articles, 1 is short communication and the rest are regular articles. The authors of these articles come from different countries namely China, India, Indonesia, Iran, Malaysia, Nigeria, Phillippines, Thailand and United Kingdom.

Articles submitted in this issue cover various scopes of Tropical Agricultural Science including aquaculture, crop and pasture production, fisheries sciences, food and nutrition development, forestry sciences, genetics and molecular biology, microbiology, plant physiology, soil and water sciences and zoology.

A regular article entitled "Improvement of Physico-Chemical Properties, Antioxidant Capacity and Acceptability of Carrot Cake by Partially Substituting Sugar with Concentrated *Nypa fruticans* Sap" discussed on the effects of nutritional, textural, antioxidant properties and acceptability of carrot cake formulated with concentrated NFS as partial replacement (0, 5, 10, 15 and 20%) for table sugar. Nutritious carrot cake with desirable physical and sensorial acceptability can be developed with the incorporation of concentrated NFS up to 20% in carrot cake. The detailed information of this article is presented on 883.

A selected regular paper from the scope of genetic and molecular biology, entitled "Response of *Cayratia trifolia* towards Pb, NaCl, Diesel And Wounding Stresses through Expression of a *CtSRG1* Gene" identified and characterized a candidate gene, named as *CtSRG1* gene, which involved in the stress response. The up- and down-regulation of this gene with the treatments of Pb, NaCl, diesel and wounding were shown through the running of qRT-PCR, respectively. Details of this study is available on page 957.

Ivan Kar Mun Chiew and the researchers summarized the major viral and bacterial pathogens of fish and crustaceans of Malaysian aquaculture industry in their review paper, which entitled "The Significance of Major Viral and Bacterial Diseases in Malaysian Aquaculture Industry". The characteristics and the consequences of diseases as well as the potential for future disease emergence in these aquatic animals were discussed. The further details of the study can be found on page 1023.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of JTAS, who have made this issue possible.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Differentiation of Malaysian Farmed and Commercialised Edible Bird's Nests through Nutritional Composition Analysis

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ABSTRACT

The growing demand of edible bird's nest (EBN) worldwide as well as competition from neighbouring countries has made the EBN industry one of the rising industries in Malaysia with stringent exportation requirement by China. However, as majority of the EBN products in the market is in commercialised form, studies on the nutritional composition of these commercialised EBN in comparison with farmed, raw EBN are limited. The farmed EBN samples were taken from 4 different regions of Malaysia: Perak (central), Kelantan (eastern), Johor (southern) and Sarawak (west Borneo) while the commercialised sample was obtained from a local drug store. Proximate, amino acid and elemental composition were performed on these EBNs. Farmed EBNs mainly comprised protein followed by carbohydrate while the commercialised EBN had similar protein and carbohydrate composition. The total collection of essential amino acid in commercialised EBN was higher (237.9 mg/g protein) compared to the farmed EBN which was between 156.81 – 236.6 mg amino acid/g protein. Among the essential amino acids, valine was found

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to be highest in both commercialised and farmed EBN. The differences between the nutritional compositions of EBNs could be due to the process of commercialisation of the EBN as well as seasonal, breeding sites and diet of the swiftlets. Farmed EBN therefore can be considered to be more nutritional due to higher protein levels.

Keywords: Amino acid analysis, commercialised EBN, edible bird's nest (EBN), elemental analysis, farmed EBN, food analysis, food composition, proximate analysis

INTRODUCTION

Edible bird's nest (EBN) is a well-known and precious delicacy among Chinese communities around the world produced by swiftlets. Swiftlets are birds (Apodidae) similar to swallows and sparrows but are not closely related. They consume a wide range of aerial insects like the other species however, they are able to fly at a higher velocity and manoeuvrability (Lim, 2007). Swiftlets weigh between 6 to 40 g which makes them relatively small in size (Ibrahim et al., 2009). They are mainly found in South East Asia countries from the coastal regions of Malaysia, Thailand, Vietnam, Islands in the Philippines and South-Eastern part of China (Aowphol et al., 2008; Phach & Oisin, 2007). Among the many species that produce EBN, *Aerodramus fuciphagus* and *Aerodramus maximus* are of commercial interest as the nest produced by these swiftlets are most pure hardened cement with minor trails of feathers and contaminants (Zukefli et al., 2017).

EBN has been eaten in traditional Chinese cuisine from as far back as the Tang Dynasty (907 AD). Traditionally EBN is prepared by double boiling with rock sugar and is known to maintain general health and youthful looking skin. It has been proven in many of the research studies on the health benefits of the EBN such as the proliferative effects of the corneal keratocytes and human adipose-derived stem cells (hADSCs). EBN was also found to inhibit the hemagglutination of the human erythrocytes caused by the influenza A virus. EBN extract also appeared to promote bone strength and improvement of skin complexion (Haghani et al., 2016).

The history of the swiftlet industry in Malaysia is unique and has grown since the 18th century where most of the nests were collected from the caves. With rapid urbanisation, the availability of the swiftlet nesting site has reduced and therefore the use of buildings that imitate cave-like environment as a swiftlet nesting site has been promoted this is called swiftlet farming. This industry has grown sizeable over the years and has a market value of USD 1000 to USD 10 000 per kilogram(Chua & Zukefli, 2016; Looi & Omar, 2016). The high price of the EBN has led to the widespread of counterfeiting and adulteration of the EBN. The commonly used materials include *Tremella* fungus, gum karaya, red seaweed, gelatine, agar and starch (Ma & Liu, 2012).

In contrast to the rapid growth of the swiftlet industry, comparative scientific investigation on the nutritional and medicinal properties between farmed, unprocessed

EBN and commercialised, ready-to-eat EBN is not as rapid. It had been reported that the major macro nutrients components were found out to be carbohydrates and proteins with 10-27% and 40-60% of the total mass respectively (Ma & Liu, 2012). Additionally, EBN is a mucin glycoprotein; with a polypeptide backbone and polysaccharides chains with a molecular weight between 40-130 kDa (Shim et al., 2016). All essential amino acids have been reported to be present in EBN with two of amino acid markers were suggested to differentiate between house and cave EBN; namely tyrosine and glutamic acid (Seow et al., 2016). Mineral composition of different types of EBN has also been reported (Marcone, 2005) and amount of minerals in EBN is highly correlated with the higher ash content in the sample (Chua & Zulkefli, 2016). However, to ease EBN consumption, many EBN products have been prepared in a more commercialised manner. Adulteration of these EBN products with gum karaya, red seaweed, *Tremella* fungus may alter its nutritional composition and therefore, this paper plans to investigate the nutritional composition of both commercialised and farmed EBN collected from various parts of Malaysia.

MATERIALS AND METHOD

Edible Bird's Nest Preparation

Farmed EBN in this paper is defined as house, uncleaned, raw EBN while commercialised EBN is defined as commercially cleaned, processed nests which are being sold to the public for consumption. Around 30 pieces of farmed nest per region were collected from several regions throughout Malaysia to represent different regions of Malaysia namely Perak (central), Kelantan (eastern), Johor (southern) and Sarawak (west of Malaysia) before pooled to depict each of the regions. These nests were cleaned by soaking in distilled water and manually to remove dirt, feathers and other foreign materials with a use of forceps. After cleaning process, the nests were air dried before being ground into powder using a mortar and pestle. The finely grounded EBN was then filtered through different size filters to remove left over feathers and other foreign materials. The commercialised EBN collected from swiftlet houses of a reputable brand claimed to be originated from Sarawak was obtained from a local pharmacy store. Conventional cleaning process for this type of EBN consisted of separating the feathers and dirt with immersion of the nests in high grade reverse osmosis water. Separation of large feathers and other impurities were achieved by using forceps and for smaller feathers, vegetable oil was used to float the feathers. Before drying in the oven, the separated strands of EBN and broken filaments were molded into leaf shape depending on the producer's preference. Upon reception, these samples were finely ground as described before and labelled accordingly before being kept in an air-tight bottle until further analysis. About 15 pieces of commercialised EBN were utilised in this study.

Proximate Composition Determination

The determination of moisture, protein, fibre, ash and fat contents of the ground EBN samples were performed following the official methods of the Association of Official Analytical Chemistry (AOAC, 2005). The moisture content of the samples was determined according to AOAC by oven drying (method 952.45). The dried samples from the previous analysis were used to determine the ash content by inserted into a muffle furnace at 550 ˚C for 5 h. The micro Kjeldahl method was used to determine the crude protein content in the samples, using 6.25 as a conversion factor (method 945.16). Fibre was determined after digesting a known weight of a fat free sample in refluxing with 1.25% sulfuric acid and 1.25% sodium hydroxide (method 926.09). The crude fat content was measured for the extracted lipid fraction of the EBN samples (Igwe et al., 2007). The crude carbohydrate content was calculated by deducting the sum of protein, fat, fibre and ash from 100%).

Elemental Analysis

Two grams of EBN sample was burned at 550°C for 5 h until no black particle was present. The sample was then added with concentrated hydrochloric acid (HCl) and was boiled until near dryness. The residue was then resuspended in 2 N HCl and filtered through a filter paper and was made up to 100 mL with deionised water. The adsorption of the solution was measured using an Atomic Absorption Spectrometer (AAS) according to the AOAC method 965.09 (AOAC, 2005).

The standard solution was prepared by dissolving 1.249 g of $CaCO_3$ in a minimum amount of 3 N HCL and diluting with deionized water to 1 L. The final stock solution was prepared by diluting 50 mL of the previous solution to 1 L with deionized water. One gram of pure zinc metal was dissolved in 10 mL of 6 N HCl and diluted with deionized water to 1 L. The standard was then diluted with dilution factors of 5x, 20x and 50x. The absorption of the solution was measured using an atomic absorption spectrophotometer (AAS) according to AOAC method 965.09 (AOAC, 2005).

Amino Acid Analysis

The amino acids content of the samples was determined according to Waters™ PICO.TAG™ method (P/N 88131). An internal standard (AABA) was prepared by dissolving 0.2578 g using 0.1 M HCl and adjusting the volume to 1 L. Mobile phase A contained 0.1 M ammonium acetate at pH 6.5, and mobile phase B had 0.1 M ammonium acetate with acetonitrile and methanol (44:46:10) at pH 6.5. The pH was adjusted using acetic acid. The preparation of redrying solution was performed by mixing methanol, water and triethylamine with a ratio of 2:2:1, and the derivation reagent contained phenylisothiocyanate (PITC), deionized water, trimethylamine and methanol with a ratio of 1:1:1:7.

For analysis, a 4/x g of sample was weighed, where x represented the percentage of protein in the respective samples, which were determined earlier. The samples were placed in test tubes with covers, and 15 mL of 6 N HCL was added to the sample and vortexed. The test tubes were put in an oven at 110°C for 24 h. After cooling, 10 mL AABA (internal standard) was added to the test tubes. The hydrolysed samples were poured into 50-mL volumetric flasks and brought to volume with deionized water, then filtered through filter paper (0.2 µm cellulose membrane filter). A 10 µL aliquot of the hydrolysed sample was put into a Durham tube. The same amount of mixed amino acids standard was also inserted into another Durham tube. The tubes were dried under vacuum for 30 min, and 20 µL of redrying solution was added to each tube before mixing the mixtures. The tubes were vacuum dried for another 30 min and were then mixed with 20 µL of derivatization reagent and vortexed. The tubes were left at room temperature for 20 min and were vacuum dried for 30 min until dryness. High pressure liquid chromatography (HPLC) with an RP 18 column (3.9 mm x 15 cm) was used to identify and quantify the amino acids. Before injection into the HPLC, the samples and standards were mixed with 100 µL of mobile phase A and vortexed for 15 min. The injection volumes of the standard, blank and samples were 8, 8 and 20 µL, respectively.

Statistical Analysis

All variables were tested for normality by applying the Kolmogorov-Sminov test and results were expressed as mean ± standard deviation (n=2). The data was statistically treated by using the Fisher least significant different method and $p < 0.05$ considered to be statistically significant. The statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc. USA) and Minitab 17 Statistics software (USA).

RESULTS AND DISCUSSION

Proximate Analysis

The proximate composition of the EBN collected different regions of Malaysia is shown in Table 1. From Table 1, there was significant difference in the moisture content of EBN from Johor, Kelantan, Perak, Sarawak with the commercialised EBN. The EBN from Johor showed the highest moisture with 17.7% whereas the commercialised EBN had the lowest

Table 1
Proximate analysis of farmed EBN samples collected from various regions of Malaysia in comparison to commercialised EBN

Origin of EBN	Proximate composition (% dry matter, n=2)					
	Moisture	Ash	Fibre	Crude fat	Crude protein	Carbohydrate
Commercialised	$0.9{\pm}0.01^{d}$	$3.9{\pm}0.04^{c}$	$0.5{\pm}0.03^{b}$	$0.4{\pm}0.03^{b}$	$46.6{\pm}2.35^{b}$	$47.7{\pm}2.39^{a}$
Johor	$17.7{\pm}0.04^{a}$	$5.1{\pm}0.1^{b}$	$0.1{\pm}0.01^{c}$	$0.3{\pm}0.01^{c}$	$55.3{\pm}0.45^{a}$	$21.5{\pm}0.41^{b}$
Kelantan	$15.3{\pm}0.23^{c}$	$5.0{\pm}0.07^{b}$	$0.0{\pm}0.00^{c}$	$0.3{\pm}0.01^{c}$	$56.3{\pm}0.34^{a}$	$23.1{\pm}0.19^{b}$
Perak	$17.6{\pm}0.01^{ab}$	$5.4{\pm}0.02^{a}$	$0.1{\pm}0.01^{c}$	$0.3{\pm}0.01^{c}$	$54.2{\pm}0.18^{a}$	$22.4{\pm}0.19^{b}$
Sarawak	$16.7{\pm}0.01^{b}$	$5.4{\pm}0.09^{a}$	$0.7{\pm}0.11^{a}$	$1.9{\pm}0.04^{a}$	$55.2{\pm}0.83^{a}$	$20.1{\pm}0.98^{b}$

* Means in category column that do not share a letter are significantly different

content of 0.9%. The amount of moisture contents found in commercialised EBN was significantly lower than in all farmed EBN while EBN from Kelantan appeared to be having the least moisture content in comparison to EBN collected from Johor, Perak and Sarawak. Although Ma and Liu (2012) reported to have lower range of moisture content (7.5 - 12.9%), Saengkrajang et al. (2013) had similar range of moisture content as reported in this study (17.8 - 24.3%).

The protein content was found to be the highest macro component found in farmed EBN originated from Johor, Kelantan, Perak and Sarawak with 55.3%, 56.3%, 54.2% and 55.2%, respectively except for commercialised EBN with 46.6%. However, no significant difference in protein composition was found between different types of farmed EBN. In contrast, crude protein levels found in this study were lower than other studies (Norhayati et al., 2010; Saengkrajang et al., 2013) but higher than local ones reported by Nurul Huda et al. (2008). The differences of protein content in the EBN could be caused by the variation in climate conditions (Hun et al., 2015). Proteins found in EBN have been proven to have many potential benefits such as epidermal growth (Ma & Liu, 2012) and inhibition of influenza viruses (Guo et al., 2006).

Carbohydrate contents of the farmed EBN were not significantly different from each other with an average value of 27 % but it was significantly higher in the commercialised EBN compared to the farmed EBN from different regions. The lower carbohydrate content from the farmed EBN could also be due to cleaning and washing processed used to clean EBN which is different from commercialised cleaning method for commercialised EBN. The carbohydrate content of farmed EBN was found to be similar (20.1-23.1%) with previous reports which ranged between 10.63-31.4% (Ma & Liu, 2012; Saengkrajang et al., 2013). The unusually high amount of carbohydrate in commercialised EBN could be due to the presence of adulterant such as gum karaya, which predominantly consists of carbohydrate (Marcone, 2005).

The fibre was found to be very minute in every EBN examined between 0.1 - 0.7 % while none was detected in Kelantan EBN. The presence of fibre was only reported in a study by Saengkrajang et al. (2013) and it was in accordance with farmed EBN (with an exception of Sarawak origin) in this study as fibre being the smallest constituents in EBN. Crude fat was found to be the highest in composition in Sarawak EBN followed by commercialized EBN. The crude fat content detected (0.3 – 1.9 %) was lower compared to the studies of EBN done in provinces in Indonesia (2.3 – 9 %) but in close margin to the other studies done in Penang, Malaysia (0.2 - 2.5 %) (Marcone, 2005; Nurul Huda et al., 2008) Crude fat was found to be the highest in Sarawak EBN which might be due to the differences in humidity level of Sarawak's caves which contributed to the hydrolytic cleavage of the triacylglycerol of EBN (Marcone, 2005).

The amount of ash found in commercialised EBN was lower than in farmed EBN but was within the range as reported by previous studies (2.1 - 7.4%) for farmed EBN (Ma & Liu, 2012; Saengkrajang et al., 2013).

Variations found in the proximate composition of EBN were contributed by the locations (regions) as good feeding environment and affluence of insects serve as indicator of sustainable swiftlet populations.

Amino Acid Analysis

The amino acid composition of farmed and commercialized Malaysian EBN is presented in Table 2 with the total collection of essential amino acids collected from the farmed EBN is between 156.81 – 236.6 mg amino acid/ g protein while the commercialised EBN has a total of 237.9 mg amino acid / g protein. Among all essential amino acids, valine was found to be the highest in all farmed EBN samples including commercialised EBN while methionine was the very least non-essential amino acid in all types of EBN samples. As for the non-essential amino acids, cysteine was found to be the highest in commercialized EBN but appeared to be the second least amino acid in all farmed EBNs. The most predominant amino acids found in farmed EBN collected from different regions of Malaysia is aspartic acid which was the highest in Kelantan (53.7 mg amino acid/g protein) compared to Johor, Perak, Sarawak with 49.7, 49.3 and 36.8 mg amino acid/g protein respectively.

The total collection of essential amino acids of farmed EBN in this study was far higher than what was found in Thailand (15.9 to 31.6 mg amino acid/g protein) (Saengkrajang et al., 2013). Aspartic acid was found to be the most predominant amino acids in farmed EBN collected from different regions of Malaysia and being one of the non-essential amino acids found in EBN, these findings were in agreement with Saengkrajang et al. (2013) in which glutamine, among non-essential amino acid which was the most predominant amino acids found in Thailand's EBN. Aspartic acid is made from glutamic acid and has a role in neurotransmission or neuromodulator (D'Aniello, 2007). Glutamic acid was also considered to be one of the markers to differentiate house from cave EBN (Seow et al., 2016).

Conversely, cystine was the most predominant essential amino acid in commercialised EBN and this sulphur-containing amino acid are known to be the most potent modulators in lipid metabolism with beneficial functions against arteriosclerosis and metabolic syndrome (Oda, 2006). Adulterated EBN (white) was said to have less phenylalanine and tyrosine in which it produced less intense yellow-red reaction during xanthoproteic acid test (Marcone, 2005). However, the level of both amino acids was almost similar between commercialized and farmed EBN in this study.

The amount and composition of the amino acid found in the EBN might directly relates to the diet and environment of the

Table 2
Amino acid composition (mg amino acids/g protein) of farmed EBN samples collected from various regions of Malaysia in comparison to commercialized EBN

Origin of EBN	Amino acid (mg/g protein;n=2)							
	Histidine	Threonine	Valine	Methionine	Isoleucine	Leucine	Phenylalanine	Total essential amino acid
Commercialized	24.10±2.20	25.64±1.16	39.69±1.09	9.23±3.18[a]	20.24±1.83	38.55±0.65	38.78±0.95	237.9
Johor	19.14±0.45	38.74±0.88	40.84±0.05	3.60±0.23[ab]	17.99±0.53	36.28±0.31	35.19±0.05	211.56
Kelantan	20.95±0.24	41.89±0.33	41.11±0.21	3.23±0.09[b]	18.21±0.07	37.88±0.09	36.96±0.34	219.46
Perak	18.66±1.07	39.65±0.74	40.80±0.11	3.68±0.1[ab]	17.47±0.07	36.51±0.05	35.56±0.26	210.62
Sarawak	12.55±3.61	20.55±6.36	25.33±7.40	4.31±1.68[ab]	11.35±2.98	22.89±5.92	18.92±4.69	232.83

Origin of EBN	Amino acid (mg/g protein;n=2)								
	Aspartic Acid	Glutamic Acid	Serine	Glycine	Arginine	Alanine	Cystine	Total non-essential amino acid	Total amino acid
Commercialized	74.86±8.57	53.19±0.73	43.37±2.77	20.065±0.44	51.44±0.27	19.17±0.87	126.58±14.21[a]	388.69	626.59
Johor	49.78±0.20	41.17±1.97	38.79±0.92	18.43±0.77	33.92±0.42	13.91±0.51	5.41±0.68[b]	201.41	412.97
Kelantan	53.79±0.85	43.42±0.94	40.99±0.16	21.01±0.16	35.42±0.20	16.65±0.37	5.53±0.5[b]	216.81	436.27
Perak	49.38±0.01	42.52±0.01	38.13±0.16	19.73±0.55	34.06±0.38	14.67±0.31	4.42±0.89[b]	202.91	413.53
Sarawak	29.73±10.01	28.31±9.46	27.19±9.29	11.69±3.40	20.95±7.12	11.35±3.28	4.86±2.38[b]	245.47	478.3

* Means in the category column that do not share a letter are significantly different
**Means in the category column that do not have a letter are not significantly different

swiftlets itself. Past research have shown that fatty acid content in the EBN relates to the diet of the insects that the swiftlet preys on (Chua et al., 2014). This diet would likely affect the amount and composition of the amino acid in the EBN itself. Nevertheless, EBN was found generally low in essential amino acids composition and not a good candidate as a sole source of protein in human diets (Seow et al., 2016).

Mineral Content

The mineral levels detected in different types of EBN is shown in Table 3 and expressed in mg / g of EBN. As the amount of ash content in the commercialised EBN and the farmed EBN is similar, the mineral content found in the samples are also similar. According to Table 3, the mean calcium content (Ca) was the highest in both commercialised and farmed EBN collected from Sarawak followed by iron (Fe) and potassium (K). Manganese (Mn) was the least mineral component found in all types of EBN in this study. In agreement with Saengkrajang et al. (2015), Ca content was the highest in both the commercialised EBN and farmed EBN and Mn was the least mineral compound found in all EBN in this study. This can be explained due to different locations where the EBN were collected as insects (food source for swiftlets) that live near the seashore may constitute high content of Ca and other minerals such as sodium (Na), magnesium (Mg) and potassium (K) (Lee et al., 2007). Differences in the environmental conditions in which EBN were collected in this study has contributed to the discrepancy in the Ca level as reported by Seow et al. (2016) that Ca content was slightly more in the cave EBN in comparison to farmed EBN collected from Johor, Kelantan and Perak. This finding was in agreement with Nurul Huda et al. (2008) and Saengkrajang et al. (2015) with its vital role in regulating nerve and muscle function (Soetan et al., 2010). It is believed that the Na content of their nest (swiftlet saliva) was correlated with the Na content in marine aerosol (water droplets in the air) through different drinking behaviour of swiftlets (capturing water droplets in the air) (Seow et al., 2016).

It can be concluded that differences in elemental profile of different types of EBN could be contributed to availability of food and types of swiftlets (white or red

Table 3
Elemental composition of farmed EBN samples collected from various regions of Malaysia in comparison to commercialised EBN

Origin of EBN	Content of elements (mg/ g dry matter; n = 1)							
	Copper	Magnesium	Manganese	Potassium	Sodium	Calcium	Iron	Selenium
Commercialised	1.6	46.8	1.4	140.8	125.8	412.8	148.6	38.6
Johor	10.8	48.2	2	10.92	127	ND	62.8	ND
Kelantan	7.2	47.6	1.6	81.2	120.6	ND	70.8	ND
Perak	11.2	47.4	24.6	80.8	119.6	ND	434.8	ND
Sarawak	6.6	55.8	1.3	205.1	149.1	2343.3	282.3	ND

*ND means not determined

nest swiftlets). Although these minerals are important micronutrients in human diets, excessive intake of minerals can be harmful to health (Norhayati et al., 2010).

CONCLUSION

Based on the results obtained from this study, farmed EBN collected from Malaysia (Perak, Kelantan, Johor, Sarawak) were found to have 55% of protein content in comparison to the commercialised EBN which had 46.6% of protein content. Both types of EBN in this study had a high composition of amino acids with cysteine being the predominant amino acid in commercialised EBN while aspartic acid was the main essential amino acid in all farmed EBNs. Calcium was the most abundant EBN mineral in all EBN samples while manganese was the least abundant EBN mineral found in all EBN. As the farmed EBN has a higher protein level it was believed to be more nutritional and beneficial to consume as compared to the commercialized EBN. The nutritional composition of EBN might be affected by the process of commercialisation of EBN for human production as well as the seasonal, breeding sites and diet of the swiftlets. (Saengkrajang et al., 2013).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

Association of Official Analytical Chemists. (2005). *Official methods of analysis: Association of Official Analytical Chemists*. Gaithersburg, USA: AOAC International Press.

Aowphol, A., Voris, H. K., Feldheim, K. A., Harnyuttanakorn, P., & Thirakhupt, K. (2008). Genetic homogeneity among colonies of the white-nest swiftlet (*Aerodramus fuciphagus*) in Thailand. *Zoological Science*, *25*(4), 372–380. doi: 10.2108/zsj.25.372

Chua, L. S., & Zukefli, S. N. (2016). Review - A comprehensive review of edible birds nest and swiftlet farming. *Journal of Integrative Medicine*, *14*(6), 415-428.

Chua, Y. G., Bloodworth, B. C., Leong, L. P., & Li, S. F. Y. (2014). Metabolite profiling of edible bird's nest using gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry: RCM*, *28*(12), 1387–1400. doi: 10.1002/rcm.6914

D'Aniello, A. (2007). D-Aspartic acid: An endogenous amino acid with an important neuroendocrine role. *Brain Research Reviews*, *53*(2), 215-234.

Guo, C. T., Takahashi, T., Bukawa, W., Takahashi, N., Yagi, H., Kato, K., … Suzuki, Y. (2006). Edible bird's nest extract inhibits influenza virus infection. *Antiviral Research*, *70*(3), 140–146. doi: 10.1016/j.antiviral.2006.02.005

Haghani, A., Mehrbod, P., Safi, N., Aminuddin, N. A., Bahadoran, A., Omar, A. R., & Ideris, A. (2016). *In vitro* and *in vivo* mechanism of immunomodulatory and antiviral activity of edible bird's nest (EBN) against influenza A virus (IAV) infection. *Journal of Ethnopharmacology*, *185*, 327–340. doi: 10.1016/j.jep.2016.03.020

Hun, L. T., Wani, W. A., Tan, E, T, T., Adnan, N. A., Ling, Y. L., & Aziz, R. A. (2015). Investigations into the physicochemical, biochemical and antibacterial properties of edible bird's nest.

Journal of Chemical and Pharmaceutical Research, *7*(7), 228–247.

Igwe, C. U., Nwaogu, L. A., Ujuwondu, C. O. (2007). Assessment of the hepatic effects, phytochemical and proximate compositions of *Phyllanthus amarus*. *African Journal of Biotechnology*, *6*(6), 728-731.

Ibrahim, S. H., Teo, W. C., & Baharun, A. (2009). A study on suitable habitat for swiftlet farming. *E-Journal of Civil Engineering*, *1*(1), 1–7.

Lee, G. J., Duncan, R. R., & Carrow, R. N. (2007). Nutrient uptake responses and inorganic ion contribution to solute potential under salinity stress in halophytic seashore paspalums. *Crop Science*, *47*(6), 2504-2512.

Lim, C. (2007). *Make millions from swiftlet farming: A definitive guide*. Pealing Jaya, Malaysia: True Wealth Sdn Bhd.

Looi, Q. H., & Omar, A. R. (2016). Swiftlets and edible bird's nest industry in Asia. *Pertanika Journal of Scholarly Research Reviews*, *2*(1), 32–48.

Ma, F., & Liu, D. (2012). Sketch of the edible bird's nest and its important bioactivities. *Food Research International*, *48*(2), 559–567. doi: 10.1016/j.foodres.2012.06.001

Marcone, M. F. (2005). Characterization of the edible bird's nest the "Caviar of the East". *Food Research International*, *38*(10), 1125–1134. doi: 10.1016/j.foodres.2005.02.008

Norhayati, M. K. J., Azman, O., & Wan Nazaimoon, W. (2010). Preliminary study of the nutritional content of Malaysian edible bird's nest. *Malaysian Journal of Nutrition*, *16*(3), 389–396.

Nurul Huda, M. Z., Zuki, A. B. Z., Azhar, K., Goh, Y. M., Suhaimi, H., Hazmi, A. J. A., & Zairi, M. S. (2008). Proximate, elemental and fatty acid analysis of pre-processed edible birds' nest (*Aerodramus fuciphagus*): A comparison between regions and type of nest. *Journal of Food Technology*, *6*(1), 39-44.

Oda, H. (2006). Functions of sulfur-containing amino acids in lipid metabolism. *The Journal of Nutrition*, *136*(6), 1666S-1669S.

Phach, N. G. Q. U., & Oisin, J. V. (2007). On an ecological form of the white-nest swiftlet *Aerodramus fuciphagus* (Aves, Apodidae) breeding in houses in Viêtnam. *Ecology Review*, *62*, 38–40.

Saengkrajang, W., Matan, N., & Matan, N. (2013). Nutritional composition of the farmed edible bird's nest (*Collocalia fuciphaga*) in Thailand. *Journal of Food Composition and Analysis*, *31*(1), 41–45. doi: 10.1016/j.jfca.2013.05.001

Seow, E. K., Ibrahim, B., Muhammad, S. A., Lee, L. H., & Cheng, L. H. (2016). Differentiation between house and cave edible bird's nests by chemometric analysis of amino acid composition data. *LWT - Food Science and Technology*, *65*, 428-435. doi: 10.1016/j.lwt.2015.08.047

Shim, E. K. S., Chandra, G. F., Pedireddy, S., & Lee, S. Y. (2016). Characterization of swiftlet edible bird nest, a mucin glycoprotein, and its adulterants by Raman microspectroscopy. *Journal of Food Science and Technology*, *53*(9), 3602-3608.

Soetan K. O., Olaiya, C. O., & Oyewole, O. E. (2010). The importance of mineral elements for humans, domestic animals and plants: A review. *African Journal of Food Science*, *4*(5), 200-222.

Zukefli, S. N., Chua, L. S., & Rahmat, Z. (2017). Protein extraction and identification by gel electrophoresis and mass spectrometry from edible bird's nest samples. *Food Analytical Methods*, *10*(2), 387–398. doi: 10.1007/s12161-016-0590-7

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Improvement of Physico-chemical Properties, Antioxidant Capacity and Acceptability of Carrot Cake by Partially Substituting Sugar with Concentrated *Nypa fruticans* Sap

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ABSTRACT

Nypa fruticans sap (NFS) or neera juice is a sugar-rich palm sap beverage widely consumed among the Malay community in Malaysia. The processed NFS has long been used as a traditional medicine to treat diabetes. Being a natural sweetener, NFS could potentially be used as a novel source to replace added sugar in bakery product. This study aimed to determine the effects of nutritional, textural, antioxidant properties and acceptability of carrot cake formulated with concentrated NFS as partial replacement (0, 5, 10, 15 and 20%) for table sugar. Results indicated that incorporation of concentrated NFS at 20% level significantly ($p<0.05$) increased the nutritional compositions of carrot cakes which recorded a higher moisture (33.31%), ash (1.47%) and dietary fiber (10.75%) content as compared with control carrot cake. In texture profile analysis, a slight decrease in hardness, springiness, chewiness and resilience were reported with increasing levels of concentrated NFS formulated in the cake. For antioxidative activities, methanolic extract of 20% concentrated NFS-incorporated carrot cake showed the highest total phenolic content (TPC) among other treatments but did not significantly differ ($p>0.05$) with TPC of 15% NFS-incorporated cake. A significantly higher ($p<0.05$) free radical scavenging effects on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power (RP) were also observed in methanolic extract of 20 % NFS carrot cake compared to control cake without NFS substitution. Sensory acceptability test revealed that carrot cake formulated with 20% concentrated NFS, though not

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significant ($p>0.05$) compared with other treatments (0 to 15%), received the highest scores for colour, texture, appearance and overall acceptability. Overall, the present study suggested that incorporation of concentrated NFS up to 20% in carrot cake to replace sugar could be an effective way to develop nutritious carrot cake with desirable physical and sensorial acceptability.

Keywords: Bakery product, functional food, natural sweetener, *Nypa fruticans*, palm sap

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder commonly presenting with episodes of high blood glucose level (hyperglycaemia) and glucose intolerance, as a result of lack of insulin, defective insulin action, or both (Sicree et al., 2006). In Malaysia, the prevalence of DM has experienced an upward trend as it remains the second most common chronic illness in the country. In a span of just a decade, there has been an 80% increase in the prevalence of diabetes. This number has exceeded the estimated prevalence of DM for the year 2025 (Letchuman et al., 2010). The increasing trend of DM seems to have linked to the continued escalation of the availability of added sugar and sweeteners (kg per capita per year) in Malaysia which has risen from 28.8 kg to 48.7 kg, or almost 70% between 1967 and 2007, according to the data from the Malaysian Food Balance Sheet (Food and Agriculture Organization [FAO], 2017). The common added sugars which include table sugar, sweeteners and jam are widely used in food industry for preparation of commercially processed products such as breads, biscuits, cakes, carbonated drinks, ice cream and local *kuih*. The main types of added sugar that are frequently used in processed foods are white refined sugars which are virtually 100 percent sucrose without additional nutrients (Insel et al., 2018).

Consumption of artificial sweeteners such as aspartame, sucralose, saccharine and neotame on the other hand has been promoted as a prevention strategy to replace added sugar. However, the health risks of artificial sweeteners consumptions are still a highly controversial topic, which have allegedly been linked to adverse effects such as cancer, weight gain and metabolic disorders (Gupta et al. 2012; Harpaz et al., 2018; Marinovich et al., 2013). Therefore, discovery of novel alternative nutrient-rich natural sweetener to replace added sugar in processed foods is currently of major interest.

The nipa palm (*N. fruticans*) is a high sugar-yielding mangrove palm tree that produces palm sap from the cut stalks of inflorescences after the removal of the floral or fruit heads (Fong, 1992; Hamilton & Murphy, 1988; Päiväke, 1996). NFS composed mainly of sucrose, glucose and fructose and it is also an abundant source of inorganic minerals, amino acids and vitamins (Aimi et al., 2013; Nguyen et al., 2016). Due to this high sugar content, the NFS has been utilised as a material of treacle, amorphous and for producing vinegar alcohol or fermented beverage called “tuba” or “soom” in the Philippines, "arak" or "tuak" in Indonesia, "toddy" in Malaysia, India and Bangladesh (Hamilton & Murphy, 1988; Päiväke, 1996).

Previous study by Sukairi et al. (2018) regarding NFS had reported that the nipa sap contained significant amount of bioactive compounds and showed a great potential in the antidiabetic properties, suitable to be used as an alternative to produce natural sugar that gave a lot of benefits. These bioactive compounds present as food components have an influence on the body, specific tissues or cells. Bioactive compounds such as polyphenols, carotenoids, tocopherols and phytosterols are distinct from nutrients in which they are not essential and, currently, no daily intake value is recommended (Gibney et al., 2009). However, they establish beneficial effects such as antioxidant activity, inhibition or induction of enzymes and gene expressions (Correia et al., 2012). The majority of dietary antioxidant compound such as polyphenols potentially retard carbohydrate digestion by delaying the activities of the enzymes α-amylase and α-glucosidase which are responsible for the digestion of carbohydrate and absorption of glucose in the digestive tract, respectively (Ali et al., 2006; Bhandari et al., 2008). The most common polyphenols that exhibit α-amylase inhibition characteristics are flavonoids. Flavonoids act as potent non-competitive inhibitors to the enzyme α-amylase by hydrophobic interactions (Yuan et al., 2014). This inhibition causes the delaying digestion of the monosaccharides in the intestine, lowering blood glucose level and reducing the potential of DM.

According to Malaysian Adult Nutrition Survey (MANS), consumption of bakery products such as biscuits, breads, local *kuih* has appeared in the list of top ten daily consumed foods (Norimah et al., 2008). Therefore, being a popular, easily available and convenient food, bakery products have a wide potential in developing functional foods by incorporation or partial replacement for sweetener to improve the overall nutritional quality of the bakery products. In this study, plain carrot cake was chosen as a baked-based product model for its healthier content, to partially substitute the table sugar for concentrated NFS up to 20% as a reduction of 20% sugar is the most acceptable and could be achieved without difficulty as reported by Shukla (1995). The objective of this study was to determine the effect of partial replacement of sugar with concentrated NFS on nutritional compositions, textural properties, antioxidative activities and sensorial acceptability of carrot cakes.

MATERIALS AND METHODS

Materials

Four batches of fresh NFS sample were collected and supplied from the same nipa farm at different months (May, July, September and November in the year 2018) from Pak Su Kemumin Enterprise, Kampung Kemumin, Kota Bharu, Kelantan state of Malaysia. The sap was acquired by cutting the stalk of matured nipa fruit and sap was collected four times daily to avoid spontaneous fermentation by minimising the chance of contamination through direct contact with the environment. The end cut of the stalk was wrapped with sterile plastic bags to maintain hygiene. The collected sap was pooled together and immediately delivered in a cooler box with ice at 0^{o}C to the laboratory located at School of Health Sciences, Universiti Sains Malaysia, Kelantan. Fresh NFS was then filtered using cream seperator (Motor Sich-100-18 Separator, Ukraine) to remove the sedimentation.

Experimental Design

The filtration, processing and chemical analyses of fresh NFS sample were done on the same day of sample delivery to maintain the quality of fresh NFS sample. Concentrated NFS sample was obtained after the processing of NFS and to be substituted readily in the carrot cake formulations.

There were control and four experimental carrot cake formulations. The control formulation was the carrot cake without substitution of concentrated NFS (0%), whilst the experimental formulations were the carrot cakes incorporated with 5, 10, 15, 20% concentrated NFS to partially replace sugar. This study compared the different carrot cake formulations in terms of the nutritional values, textural properties, antioxidant capacities and sensorial acceptability.

Processing of NFS

The dehydration technique and storage of neera were adapted with modifications from the techniques described by Naknean et al. (2013). Forty grams of the filtered sap were dehydrated in a thermal dehydrator (Anywin FD770, China) at 62^{o}C for an overnight (15 hours) to evaporate the moisture content. After heated, concentrated NFS samples were stored in a chiller at -4^{o}C prior to analysis and further use.

Chemical Analyses of NFS

The measurement of pH was done on NFS samples using HANNA pH 211 microprocessor pH meter (USA) according to Association of Official Analytical Chemists [AOAC] (2012). Calibration was accomplished employing buffer solutions at pH 7.0 and 4.0. The quality of fresh NFS sample was monitored prior to analysis by measuring its pH, if a fresh NFS sample has an initial pH below 4.0, the fresh NFS will be discarded as fermentation has possibly occurred in the sample. According to Aimi et al. (2013), pH at about 3.6 indicates that the palm sap is in the fermented stage in which the fermentation is dominated by yeasts and lactic acid bacteria. Total soluble solids of samples as ^{o}Brix were measured using hand refractometer (Atago 3851 PAL-BX/RI, Japan). The sugar content of NFS was analysed with the Boehringer Mannheim/ R Biopharm 10 716 260 035 sugar analysis kit for enzymatic analysis of sucrose, glucose and fructose according to the manufacturer's instructions. Moisture of NFS samples was analysed according to AOAC methods (AOAC, 2000).

Carrot Cake Preparation

Formulations of plain carrot cake for this study were adapted from a well-known baking site (Jaworski, n.d.) with modification and listed in Table 1. Concentrated NFS was incorporated into the formulation to substitute sugar by 5, 10, 15 and 20% based on sugar weight (g) as in control formulation (0%). To begin with, the dry ingredients: wheat flour, cinammon ground, baking powder, baking soda were filtered and mixed evenly. In another bowl which contained the dry ingredients, eggs were mixed together with the sugar or concentrated NFS at speed 3 using electric hand mixer (KHIND HM 200, Malaysia) for approximately 5 minutes until the mixture turned pale and foamy. Next, corn oil was added into the egg mixture together with vanilla essence. Mixing was continued until the mixture was uniformly mixed. Next the dry ingredients were added into the mixture by gently folding the former into the latter using a spatula. Grated carrot was then added to the mixture. The combined batter was poured into a 6 inch pan. The cake was baked at 150°C for 40 minutes in the oven (ELBA 30L, Italy).

Nutritional Analysis

Proximate analysis was analysed according to AOAC methods (AOAC, 2000) to determine moisture, ash, protein, fat and total dietary fiber contents of control and NFS carrot cakes. Total carbohydrate was calculated by difference using the formula:

% Carbohydrate = 100 – (% moisture + % fat + % ash + % protein)

For mineral analysis, carrot cake samples were sent to Biochem Laboratories Sdn. Bhd., Penang to determine the sodium and potassium content of cake samples using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). Generally, ICP-OES application is a tool to detect metals and non-metals especially in solution. This tool utilises Argon gas to move the sample vapour into a chamber under high vacuum condition where both the samples and gas are heated such that elements will give off a characteristic wavelength of light.

Table 1
The raw ingredients used and incorporation level (%) of concentrated NFS

Ingredients (g)	Formulation (% concentrated NFS used to replace sugar) g /100 g flour				
	0% NFS (control)	5% NFS	10% NFS	15% NFS	20% NFS
Sugar	80.0	76.0	72.0	68.0	64.0
Concentrated NFS	0.0	4.0	8.0	12.0	16.0
Wheat flour	100.0	100.0	100.0	100.0	100.0
Corn oil	50.0	50.0	50.0	50.0	50.0
Egg	60.0	60.0	60.0	60.0	60.0
Ground cinnamon	2.1	2.1	2.1	2.1	2.1
Baking powder	4.2	4.2	4.2	4.2	4.2
Baking soda	2.1	2.1	2.1	2.1	2.1
Carrot	150.0	150.0	150.0	150.0	150.0
Orange zest	1.0	1.0	1.0	1.0	1.0
Vanilla essence	2.1	2.1	2.1	2.1	2.1

Physical Analysis

Textural analysis of carrot cakes was conducted instrumentally using TA.XTPlus Texture Analyser (Stable Micro Systems Ltd., Surrey, UK) which was driven by Exponent software package. The soft inner portion of cake was evaluated. Each cake was cut into 2.5 cm sided cubes, where the upper and lower crusts were eliminated. A 75-mm diameter aluminium plate (P/75) was used for compression. Probe height was initially calibrated to ensure that the travel distance of the probe can be recorded. The test was carried out under the following condition, test speed 1 mms^{-1}, strain 50%, double cycles, 5 sec interval between cycles and trigger force 5 g. The hardness (N), springiness, cohesiveness, resilience and chewiness attributes of cakes were calculated from the curves obtained.

Sample Extraction for Total Phenolic Content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

Carrot cake sample (1 g) was extracted with 100 mL methanol 80% (v/v) in conical flask (250 mL) wrapped with aluminium foil according to method suggested by Uthumporn et al. (2015) with slight modification. The mixture was then shaken overnight in an orbital shaker (Thermo Scientific Forma Incubated Benchtop Orbital Shaker Model 420, United States) at 160 rpm at 27^{o}C. Next, the mixture was poured into centrifuge tubes and centrifuged (Eppendorf MiniSpin Plus, Germany) at 420 × *g* for 30 minutes to get a clear solution. Light exposure was prevented throughout the extraction process. The extract was directly used to determine total phenolic content (TPC) and antioxidant value (2,2-diphenyl-1-picrylhydrazyl, DPPH).

Sample Extraction for Reducing Power Assay

Carrot cake sample (0.25 g) was extracted triplicate according to method described by Szawara-Nowak et al. (2017) with 5 mL methanol 67% (v/v) in conical flask wrapped with aluminium foil and shaken overnight in an orbital shaker (Thermo Scientific Forma Incubated Benchtop Orbital Shaker Model 420, United States) at 160 rpm at 27^{o}C. Next, the mixture was poured into centrifuge tubes and centrifuged (Eppendorf MiniSpin Plus, Germany) at 420 × *g* for 30 minutes to get a clear solution. The extract was dried in a fume hood overnight. Then the dried extract was dissolved in 0.2 M, pH 6.6 phosphate buffer (20 mg/mL) and was used immediately for the measurement of reducing power of cake extracts.

Total Phenolic Content (TPC)

TPC of carrot cakes were determined by using Folin–Ciocalteu's (FC) assay which was described by Singleton and Rossi (1965) with slight modification. Result obtained was expressed as gallic acid equivalent (mg GAE/ g extract).

DPPH Activity

The antioxidant capacity of the cake extracts was studied through the evaluation of the free radical-scavenging effect on the DPPH radical. The determination was based on the method proposed by Ancos et al. (2002) with modification. This method was also called as DPPH free radical scavenging assay. The DPPH radical scavenging activity was expressed as percentage of inhibition (% of inhibition).

Reducing Power (RP)

The reducing power of cake extracts was determined by the method of Oyaizu (1986) with slight modification according to Liyana-Pathirana and Shahidi (2006). Result obtained was expressed as ascorbic acid equivalent (mg ascorbic acid/ g extract).

Sensory Evaluation

The sensory evaluation of carrot cakes was carried out by 60 untrained panellists consisting of staff and students of the School of Health Sciences, Universiti Sains Malaysia. Panellists received five different formulations of carrot cakes for evaluation. The tested samples were coded with a three-digit randomly permuted number. All samples were rated according to the seven-point hedonic scales (1 = dislike extremely, and 7 = like extremely). Sensory attributes such as colour, appearance, texture, taste, flavour and overall acceptance were evaluated based on their degree of overall likeness for carrot cake.

Data Analysis

All data were subjected to one-way repeated measure analysis of variance (ANOVA) followed by Tukey HSD test to compare mean differences among the samples. Data analysis was done using IBM SPSS Statistics Version 24 (SPSS Software, Armonk, New York). Three replicates were performed for all batches of fresh and concentrated NFS samples. Also, three batches of carrot cakes were produced in this study for all measurements. Results were expressed as mean values of three replicates ± standard deviation (SD) except for dietary fiber (n=2), mineral analysis determination (n=2) and sensory evaluation (n=60). All tests were two-tailed and significance level was established at $p<0.05$.

RESULTS AND DISCUSSION

Chemical Quality of NFS

NFS sample batches were chosen to be collected on May, July, September and November in the same year in 2018 as these months represented a monsoon change in the East Coast of Malaysia. Being one of the East Coast states in Malaysia, Kelantan has faced two monsoon seasons, the Southwest Monsoon from April to September which leads to a drier weather and the Northeast Monsoon from October to March, bringing to a higher rainfall (Malaysian Meteorological Department [MetMalaysia], 2019). The monsoon change has led to the variation of chemical quality of NFS collected as can be seen in Table 2. The pH values of fresh NFS samples ranged from 4.33 on November to 4.82 on May. Preliminary studies on NFS also found that unprocessed fresh NFS has an initial pH of 4.5 - 6.5 (Aimi et al., 2013; Minh, 2014). According to Aimi et al. (2013), pH at about 3.6 indicates palm sap is in the fermented stage in which the fermenting organisms are dominated by yeasts, particularly *Saccharomyces cerevisiae* and lactic acid bacteria. According to the average rainfall monthly report by Jabatan Pengairan dan Saliran Negeri Kelantan (JPS Negeri Kelantan) (2018), the rainfall station located in Kota Bharu, Kelantan had collected the highest record of average rainfall (30.9 mm) on the month of November. This had resulted in the yield of palm sap having a significantly ($p<0.05$) higher moisture content (83.86%) on November compared to other months. According to Matsui et al. (2014), the production of palm sap from *N. fruticans* fluctuates in a year in which sugar production tends to decrease in the rainy season. Generally, when the volume of sap produced is high (high moisture content), the sugar content in sap (TSS) tends to decrease. The major carbohydrates found in fresh NFS samples analysed in this study were sucrose (115.45 to 127.03 g/L), followed by fructose (15.25 to 21.92 g/L) and glucose (5.86 to 6.71 g/L). Differences in sucrose, fructose and glucose concentrations of fresh NFS samples may be due to the variation among fruit stalks and the origin of palm trees (Matsui et al., 2014).

Traditionally, palm sap syrup is produced by evaporating the palm sap in an open pan, and heated, using a wood fired stove, until it becomes concentrated. This heat causes the formation of dark colour, known as the browning development. Browning caused by Maillard reaction and caramelization may lead to unacceptable nutritional and sensory effects in sugar-based food products and may be a limiting factor in the shelf life of products (Burdulu & Karadeniz, 2003). Dehydration technique suggested by Naknean et al. (2013) showed that evaporation heat treatment processing of palm sap at 70 to 80°C was better in retaining desired quality attributes in syrup than by heating with an open pan. According to a recent study by Yunos et al. (2018), evaporation heat treatment of palm sugar that maintained at low and controlled temperature exhibited the highest ability in ion chelating activity which related in antioxidant activity compared to freeze-drying and open pan methods. Besides, it could retain more sucrose content than further inverted compared to palm syrup heated by an open pan. This dehydration technique was also supported by Tamunaida and Saka (2011) showing that the saps treated at 70°C were higher in total sugars. In fact, in our study, the recorded temperature for the dehydration process was found to be 62°C which was lower than the recommended temperature so as to minimise the loss of bioactive compounds which might present in NFS samples. Non-enzymatic browning of palm sugar syrup during storage could also be reduced by storing at low temperature (-4°C). Chemical quality characteristics of concentrated NFS after processing at 62°C were shown in Table 3. Concentrated NFS obtained after processing was found to have a range of pH value of 5.24 to 5.34 from the four batches samples, higher than the pH of the fresh NFS. The increase of pH observed after processing of NFS might be caused by the interaction between reducing sugars and amino compounds present in NFS samples resulted in the formation of Amadori rearrangement as an effect of elevated temperature. Besides, the Strecker degradation of amino acids might contribute to the increase in pH by losing CO_2 from the acid moiety (Akochi-K et al., 1997). This indicated that the NFS contains minerals and amino acids rather than simply a pure solution of sugar. From Table 3, moisture content of all four concentrated NFS samples ranged from only 15.76 to 15.82% and were not significantly different ($p>0.05$) among the four batches. This result indicated that most of the moisture content in NFS had lost through the process of dehydration at 62^{o}C. Brix values are commonly used for approximation of sugar content in sugar-rich samples and can be defined as the grams of sucrose per 100 g of sample material, thus, a refractometer can be used to determine the carbohydrate content of the sap and/or finished syrup (Willits & Hills, 1976). As the major chemical components of NFS are simple carbohydrate and water, the combination of these two values should be close to 100% (Stuckel & Low, 1996). Combining moisture with ^{o}Brix value of concentrated NFS in this study gave values in range of 99.58% to 101.19%. This again showed that low heat-dehydration technique at 62^{o}C is an effective method in removing the water content without much sugar loss. According to reg. 132 of the Food Regulations 1985 (n. d.), molasses or syrup shall have TSS of not less than 85^{o}Brix at 20^{o}C. Under this definition, all four batches of concentrated NFS, which have a range of TSS from 85.40 to 86.63^{o}Brix, met this standard and could be directly incorporated into food. In the present study, concentrated NFS samples from batch J (July) were chosen to be further incorporated into the experimental cake formulations due to its highest value of TSS (86.73^{o}Brix) and the lowest moisture content (15.76%) obtained from the analysis compared to other batches from different months (Table 3).

Nutritional Composition of Carrot Cake

The nutritional composition of carrot cakes prepared from sugar replaced partially with concentrated NFS at different levels is shown in Table 4. The results revealed that the incorporation of concentrated NFS resulted in significantly different proportions of moisture, ash, carbohydrate and dietary fiber. Moisture content varied from 29.52 to 33.31% for carrot cakes prepared with the increasing amount of concentrated NFS to partially replace sugar. The result of this study indicated that all carrot cakes formulated with concentrated NFS recorded significantly higher ($p<0.05$) moisture content ranging from 31.60 to 33.31% for 5, 10 and 20% NFS-incorporated cake compared to control cake (29.52%). Similar results were reported by Ahmadi et al. (2011) and Majzoobi et al. (2016) to replace sugar (sucrose) with date liquid syrup in cookies and cakes. This can be related to the different solubility rate of sucrose, fructose and glucose during mixing in cake preparation. Sucrose remains mostly in the crystalline form, having lesser interaction

Table 2
Chemical quality of fresh NFS samples collected on four different months in 2018

Sample batch	Quality characteristics					
	pH	Total soluble solids (°Brix)	Moisture (%)	Sucrose (g/L)	Fructose (g/L)	Glucose (g/L)
M	4.82 ± 0.02^{a}	19.33 ± 0.06^{a}	80.25 ± 0.05^{d}	127.03 ± 0.14^{a}	21.92 ± 0.01^{a}	6.71 ± 0.03^{a}
J	4.77 ± 0.02^{b}	18.23 ± 0.06^{ab}	81.78 ± 0.04^{c}	126.07 ± 0.23^{b}	19.40 ± 0.12^{b}	6.22 ± 0.04^{b}
S	4.64 ± 0.02^{c}	18.13 ± 0.06^{ab}	82.12 ± 0.02^{b}	121.22 ± 0.41^{c}	17.38 ± 0.07^{c}	5.96 ± 0.05^{c}
N	4.33 ± 0.01^{d}	17.70 ± 0.10^{b}	83.86 ± 0.07^{a}	115.45 ± 0.25^{d}	15.25 ± 0.06^{d}	5.86 ± 0.07^{c}

Note. Four batches of NFS samples in this study were collected at different months (M=May, J=July, S=September, N=November) in the same year 2018
Each value is the mean of triplicate determinations ± standard deviation (n=3)
Mean values within the same column bearing the same subscript letter were not significantly different ($p>0.05$)

Table 3
Chemical quality of concentrated NFS samples dehydrated from fresh NFS collected on four different months in 2018

Sample batch	Quality characteristics					
	pH	Total soluble solids (°Brix)	Moisture (%)	Sucrose (g/100g)	Fructose (g/100g)	Glucose (g/100g)
M	5.34 ± 0.02^{a}	86.63 ± 0.06^{a}	15.82 ± 0.03^{a}	74.04 ± 0.08^{a}	11.52 ± 0.01^{a}	6.61 ± 0.09^{b}
J	5.31 ± 0.01^{ab}	86.73 ± 0.06^{a}	15.76 ± 0.06^{a}	72.01 ± 0.27^{b}	11.51 ± 0.01^{a}	$6.46 \pm 0.05_{b}$
S	5.27 ± 0.01^{bc}	85.43 ± 0.06^{b}	15.76 ± 0.07^{a}	70.76 ± 0.17^{c}	11.50 ± 0.25^{a}	6.84 ± 0.04^{a}
N	5.24 ± 0.03^{c}	85.40 ± 0.00^{b}	15.79 ± 0.10^{a}	71.42 ± 0.11^{bc}	11.44 ± 0.06^{a}	6.49 ± 0.02^{b}

Note. Four batches of NFS samples in this study were collected at different months (M=May, J=July, S=September, N=November) in the same year 2018
Each value is the mean of triplicate determinations ± standard deviation (n=3)
Mean values within the same column bearing the same subscript letter were not significantly different ($p>0.05$)

with water (low solubility) compared to reducing sugar (glucose and fructose). During baking, more water can evaporate readily before sugar solubilise, resulting in a drier cake as observed in control (0%). In contrast, glucose and fructose in concentrated NFS, have more interactions with water (higher solubility) by hydrogen bonding and prevent it from evaporating during baking, causing higher moisture content of carrot cakes. Concentrated NFS used in this study are composed of three main sugars: sucrose, fructose and glucose as determined earlier (Table 3) compared to refined table sugar (>99.5% sucrose). Hence, the higher the substitution of concentrated NFS, the higher the moisture content of NFS. Another component of carrot cakes which increased significantly ($p<0.05$) with every 5% substitution of concentrated NFS is ash content, which can be related to the inorganic minerals present in the palm saps used. According to Nguyen et al. (2016), palm sap contains high mineral contents such as potassium, chloride and sodium. Moreover, *N. fruticans* palm tree grows near the swampy seawater salt which directly contributes to the high content of sodium and chloride in NFS (Hamilton & Murphy, 1988).

Mineral analysis results from Table 4 showed that all formulations of carrot cake incorporated with concentrated NFS (5, 10, 15 and 20%) have a significantly higher sodium and potassium content as compared with the control carrot cake without addition of NFS. According to dietary guidelines issued by World Health Organization [WHO] (2013), adults should consume at least 3510mg of potassium and less than 2000mg of sodium per day to reduce blood pressure and risk of cardiovascular disease, stroke and coronary heart disease. A study by Mierlo et al. (2010) which collected data across 21 countries reported that the mean potassium intakes ranged from 1700mg/day (China) to 3700mg/day (Finland, the Netherlands, and Poland). This suggested that an increase in potassium intake should be recommended, especially among Asian countries. In contrast to the low potassium intake, a recent Ministry of Health survey in Malaysia (2017) reported that the mean population salt intake had achieved 3419mg of sodium per day, which is 1.7 times than the limit set by WHO. Considering the high content of both sodium and potassium in cake formulations incorporated with NFS, a balance consumption of the cake should be considered in one's daily diet so that the intake of potassium is adequate and at the same time excessive intake of sodium could be avoided.

From the results shown in Table 2, all formulations of cake showed a high percentage of total dietary fiber content ranging from 9.20 to 10.75% due to the presence of grated carrot in the cake. Interestingly, total dietary fiber content of 20% concentrated NFS-substituted carrot cake (10.75%) was found to be significantly ($p<0.05$) higher than the fiber content of control cake (9.75%). On the other hand, carrot cake containing 20% concentrated NFS, though not significant ($p>0.05$), recorded the highest fiber content

(10.75%) compared with 5, 10 and 15% NFS-incorporated carrot cake. This is in accordance with the findings of Trinidad et al. (2010) which reported that minimal processed palm sugars contained significant amount of dietary fiber, especially inulin.

For other nutrients such as fat and protein, incorporation of concentrated NFS showed no statistically difference (p>0.05) with the control cake, indicating that substituting sugar with concentrated NFS would not have a significant effect on the protein and fat contents.

Table 4
Proximate composition for different formulations of carrot cake

Component (%)	Concentrated NFS level (%)				
	0	5	10	15	20
Moisture	29.52 ± 0.23[c]	31.60 ± 0.07[b]	31.92 ± 0.42[ab]	32.37 ± 0.74[ab]	33.31 ± 0.77[a]
Protein	7.02 ± 0.06[a]	7.03 ± 0.11[a]	7.09 ± 0.09[a]	7.10 ± 0.27[a]	7.15 ± 0.06[a]
Fat	20.52 ± 1.38[a]	19.66 ± 1.36[a]	19.46 ± 0.06[a]	19.40 ± 0.56[a]	19.45 ± 0.20[a]
Ash	1.18 ± 0.00[e]	1.26 ± 0.02[d]	1.31 ± 0.02[c]	1.36 ± 0.01[b]	1.47 ± 0.01[a]
Sodium (mg/100g)[1]	6.90 ± 0.00[d]	174.00 ± 4.24[c]	176.50 ± 0.71[c]	218.50 ± 4.95[b]	253.50 ± 4.95[a]
Potassium (mg/100g)[1]	4.05 ± 0.21[d]	172.00 ± 4.24[b]	152.50 ± 3.54[c]	155.00 ± 4.24[c]	222.50 ± 3.54[a]
Carbohydrate	41.76 ± 1.67[a]	40.45 ± 1.56[ab]	40.22 ± 0.59[ab]	39.8 ± 1.58[ab]	38.62 ± 1.04[b]
Dietary Fiber	9.20 ± 0.08[b]	9.50 ± 0.25[ab]	10.24 ± 0.60[ab]	10.51 ± 0.29[ab]	10.75 ± 0.18[a]
Caloric Values[2]	379.81 ± 7.87[a]	366.82 ± 6.88[ab]	364.42 ± 1.75[b]	362.10 ± 3.67[b]	358.13 ± 2.96[b]

Note. Values are mean ± standard deviation
Mean values within the same row bearing the same subscript letter were not significantly different (p>0.05)
[1]Mineral analysis for sodium and potassium was done using ICP-OES performed by Biochem Laboratories Sdn. Bhd,, Penang
[2]Caloric values (kcal/100 g) = (4×protein) + (9×fat) + (4×carbohydrate)

Texture Analysis

Effects of substitution of sugar with concentrated NFS on the textural profile of carrot cakes were shown in Table 5. The present result indicated that there was an inverse relationship between hardness and the level of concentrated NFS. Among all the formulations, control carrot cake showed the highest value for hardness. The hardness of carrot cakes varied from 5.23 to 14.59N. Apparently, carrot cake incorporated with 20% concentrated NFS had a hardness value of 5.23N, which was significantly (p<0.05) lower than the control cake without the addition of NFS (14.59N). Yet, there was no statistically difference of hardness between 5 and 10%, as well as 15 and 20% of NFS-incorporated cake.. The large variation of hardness among the cakes might be due to the greater crystallisation rate of sucrose during cooling of cakes compared to fructose and glucose. Sugars do not just only play an important role in the overall sweetness of the cakes but also provide textural properties desirable in foods. According to Laos et al. (2007), crystallisation of pure sucrose supersaturated solution occurred very quickly. In contrast, crystallisation of sucrose in presence of glucose or fructose led to slower crystallisation. This finding could be related back to the sugar content of the concentrated NFS being performed earlier in this study (Table 3). Hence, the purer the sucrose content added to the cake, the higher the hardness value of the carrot cakes.

Resilience and springiness of the carrot cakes also showed a decreasing trend when incorporated with concentrated NFS. There was a significant reduction ($p<0.05$) of resilience in every 5% substitution of sugar with NFS. However, there was no statistically reduction in springiness by carrot cakes added with NFS up to 10%. At 15 and 20% level of NFS incorporation, springiness of cakes was 0.25 and 0.21, respectively which differ significantly ($p<0.05$) from springiness of control cake which was 0.31. Manisha et al. (2012) showed that the sugar provided a considerable part of the bulking agent by competing with starch for available water, and this could delay the onset of gelatinisation, improving the size of air bubbles due to carbon dioxide and water vapour before the cake sets. Moreover, sugar differs in their ability to delay gelatinisation, with the following sugars having the greatest impact (from least to greatest): fructose, glucose and sucrose (Brown, 2011). Refined sugar added in the control cake is more than the other cakes, therefore will have a greater effect in delaying gelatinisation, resulting in a cake with bigger air bubble size and higher volume. This could greatly affect the textural properties of carrot cakes in term of springiness and resilience. For other textural properties such as cohesiveness and chewiness, only slight differences were observed.

Antioxidant Capacity of Carrot Cake Extract

Characterization of a new food product often involves the usage of antioxidative capacity. There is a variety of *in vitro* test procedures to carry out for evaluating antioxidant activities with the samples of interest. Hence, antioxidant activity should not be concluded based on a single antioxidant test model. In our present study, three methods

Table 5
Textural properties for different formulations of carrot cake

Properties	Concentrated NFS Level (%)				
	0	5	10	15	20
Hardness (N)	14.59 ± 0.78^{a}	9.95 ± 0.58^{b}	8.63 ± 0.72^{b}	5.95 ± 0.16^{c}	5.23 ± 0.13^{c}
Springiness	0.31 ± 0.04^{a}	0.29 ± 0.31^{a}	0.28 ± 0.12^{ab}	0.25 ± 0.13^{bc}	0.21 ± 0.08^{c}
Cohesiveness	0.35 ± 0.02^{a}	0.27 ± 0.28^{b}	0.33 ± 0.13^{a}	0.26 ± 0.01^{b}	0.13 ± 0.00^{c}
Chewiness (N)	0.56 ± 0.11^{b}	0.56 ± 0.04^{b}	0.62 ± 0.14^{ab}	0.65 ± 0.08^{ab}	0.72 ± 0.04^{a}
Resilience	0.60 ± 0.21^{a}	0.49 ± 0.32^{b}	0.42 ± 0.07^{c}	0.30 ± 0.23^{d}	0.21 ± 0.30^{e}

Note. Each value is the mean of triplicate determinations ± standard deviation (n=3)
Mean values within the same row bearing the same subscript letter were not significantly different ($p>0.05$)

were chosen for evaluating antioxidant activities, which are Folin-Ciocalteu (FC) method to evaluate TPC, free radical scavenging capacity of DPPH and RP assay to determine the reducing power of bioactive compounds. These assays were selected to confirm the presence of phenolic content and antioxidant in food samples as they are simple, inexpensive and rapid (Badarinath et al., 2010). In fact, antioxidant test models vary in different respects. Hence, it is difficult to compare fully one method to another (Alam et al., 2013).

Table 6 shows the antioxidant capacity for different formulations of carrot cake incorporated with concentrated NFS. The TPC of carrot cakes prepared from sugar and concentrated NFS ranged from 0.11 to 0.34. The result obtained in this study indicated that TPC in carrot cake extract increased proportionally with the level of NFS added in the formulations in the following order: 0% < 5% < 10% < 15% < 20%. Carrot cake extract substituted with 20% concentrated NFS was found to have the highest TPC content (0.14 mg GAE) compared to the control carrot and other cake extracts of different formulations. However, carrot cake incorporated with 20% concentrated NFS was not significantly differed ($p>0.05$) with 15% concentrated NFS-incorporated cake. The high TPC in carrot cake formulated with concentrated NFS was mainly due to the presence of a large amount of polyphenol compound in concentrated NFS that is being substituted for sugar in the carrot cake. It is possible to hypothesise that a high content of total phenolic compounds is likely present in NFS as a result of there being no purification process applied during its manufacture, whereas refined sugar is produced by employing additional steps which remove colour and any other non-sugar components. According to Sukairi et al. (2018), NFS sample reported to highly inhibit α-amylase enzyme due to the presence of phenolic compound, for example phenolic acids and flavonoids. The phenolic compounds work by inhibiting α-amylase activity, causing a retardation in the intake of monosaccharide in the body.

From Table 6, control carrot cake extract showed a significant ($p<0.05$) lower percentage of scavenging or inhibition of DPPH free radical activity compared to 10, 15 and 20% of NFS-incorporated carrot cake extract. However, substitution of 5% concentrated NFS carrot cake extract did not differ significantly ($p>0.05$) from the control cake extract in term of DPPH radical scavenging activity. The 20% level of concentrated NFS-incorporated cake extract showed the highest scavenging activity towards DPPH (3.89% of inhibition). Both TPC and DPPH demonstrated identical trends in the comparison of the antioxidant activities in which the presence of polyphenols in concentrated NFS play a significant role in contributing to the overall phenolic content of the cake extract. The antioxidants present in the phenolic fractions were able to reduce the violet colour, stable 1,1-diphenyl-2-picrylhydrazyl radical to the yellow colour 1,1-diphenyl-2-picrylhydrazine. Previous studies have also presented positive correlations between the quantity of phenolic compounds and the DPPH free radical scavenging effect

(Kedare & Singh, 2011; Liu et al., 2009). This finding is also in accordance with the result from Sukairi et al. (2018) which reported that higher scavenging activity was observed as the concentration of NFS sample was increased, showing once again that this natural sweetener has a positive effect on the enhancement of antioxidant activity in carrot cake samples.

Another effective method to determine an extract's antioxidant properties is the reducing power (RP) assay. As shown in Table 6, the reducing power assay showed a pattern of oxidant strength identical to the TPC and DPPH assays. The reducing power of carrot cake extract increased as the substitution of sugar with concentrated NFS becomes higher, though the increment was not significant ($p>0.05$) as could be seen between 0 and 5%, as well as 15 and 20% level of concentrated NFS incorporation. The reducing power was the highest when sugar was replaced with 20% concentrated NFS in carrot cakes while the least reducing power was shown to be the control cake without any incorporation of concentrated NFS. The presence of reducers (antioxidant) in the concentrated NFS used in the cake formulation caused the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration. Again, this confirmed the presence of antioxidative compounds in the concentrated NFS sample used which directly led to an increase in the antioxidative activities of carrot cake extract when NFS was added into the carrot cakes.

Sensory Acceptability

Table 7 shows the sensory evaluation scores for carrot cakes incorporated with concentrated NFS. The result showed that the scores of all sensory attributes were in a range of 4.27 to 5.27. The present sensory data showed that all carrot cakes formulated with concentrated NFS (5, 10, 15 and 20%) were not significantly different ($p>0.05$) compared to the control cake for all attributes. Among all the carrot cake formulations, 20% concentrated NFS-incorporated cake received the highest scores for all sensory

Table 6
Antioxidant capacity of carrot cake samples determined against TPC, DPPH and RP assays

% of concentrated NFS incorporated carrot cake	Antioxidant Capacity		
	TPC (mg GAE/ g)	DPPH (% of inhibition)	Reducing power (mg ascorbic acid/g)
0	0.11 ± 0.01^{d}	2.90 ± 0.19^{b}	1.18 ± 0.01^{b}
5	0.12 ± 0.01^{c}	2.92 ± 0.19^{b}	1.18 ± 0.01^{b}
10	0.13 ± 0.01^{bc}	3.52 ± 0.19^{a}	1.27 ± 0.06^{ab}
15	0.13 ± 0.02^{ab}	3.56 ± 0.12^{a}	1.32 ± 0.03^{a}
20	0.14 ± 0.00^{a}	3.89 ± 0.07^{a}	1.34 ± 0.07^{a}

Note. Each value is the mean of triplicate determinations ± standard deviation (n=3)
Mean values within the same column bearing the same subscript letter were not significantly different ($p>0.05$)

attributes (colour, texture, appearance and overall acceptance) except for taste and flavour. On the other hand, carrot cake incorporated with 15% concentrated recorded 5.18 and 5.10 score values for taste and flavour, respectively. These values are the highest among treatments but did not show significant ($p>0.05$) differences when compared to other cake formulations (0, 5, 10 and 15%). The result also showed that carrot cakes containing 20% concentrated NFS received the highest score for overall acceptance (5.10) followed by carrot cakes containing 15% NFS (5.08). The result obtained indicated that consumers prefer 20% concentrated NFS to be incorporated in carrot cakes. Besides, consumers also preferred the 15% concentrated NFS-incorporated carrot cake in terms of taste and flavour. This data indicated that consumers generally accept the carrot cakes prepared with concentrated NFS up to 20% of sugar replacement.

Table 7
Sensory acceptability of different formulations of carrot cake

Properties	Concentrated NFS level (%)				
	0 %	5 %	10 %	15 %	20 %
Colour	5.27 ± 1.25^a	5.07 ± 1.34^a	4.87 ± 1.50^a	5.13 ± 1.41^a	5.27 ± 1.39^a
Appearance	4.82 ± 1.10^a	4.72 ± 1.14^a	4.72 ± 1.25^a	4.83 ± 1.26^a	5.00 ± 1.30^a
Texture	4.65 ± 1.29^a	4.28 ± 1.41^a	4.28 ± 1.50^a	4.55 ± 1.51^a	4.72 ± 1.40^a
Taste	4.70 ± 1.28^a	4.82 ± 1.37^a	4.52 ± 1.55^a	5.18 ± 1.10^a	5.03 ± 1.39^a
Flavour	4.80 ± 1.31^a	4.60 ± 1.30^a	4.27 ± 1.57^a	5.10 ± 1.02^a	5.00 ± 1.35^a
Overall Acceptance	4.87 ± 1.13^a	4.87 ± 1.27^a	4.62 ± 1.34^a	5.08 ± 1.01^a	5.10 ± 1.13^a

Note. Each value is the mean of determinations ± standard deviation (n=60)
Mean values within the same row bearing the same subscript letter were not significantly different ($p>0.05$)
(Score 1 = dislike extremely, score 7 = like extremely)

CONCLUSION

The addition of concentrated NFS up to 20% to partially replace sugar in carrot cake formulation resulted in a significant increment of ash content compared to other cake formulations. Carrot cake containing 20% concentrated NFS also recorded significantly higher ($p<0.05$) total dietary fiber content at 10.75g/100g as compared with control carrot cake with 0% NFS at 9.20g/100g. Texture profile analysis revealed that there was a decreasing trend of hardness, springiness and resilience in carrot cakes with the increasing levels of concentrated NFS substituted for sugar. TPC of carrot cake showed the highest value when 20% concentrated NFS was formulated in the cake but did not significantly ($p>0.05$) differ with 15% concentrated NFS-incorporated cake. The presence of antioxidant as a result of enhancement by concentrated NFS was further proved by the radical scavenging effect of DPPH and RP assay, in which a significantly ($p<0.05$) higher percentage of inhibition and reducing power was observed in carrot cake incorporated with 20% concentrated NFS, comparing with the control cake. Sensory evaluation indicated that carrot cakes incorporated with 20% concentrated NFS, though not significant ($p>0.05$) differ among all formulations, received the highest scores for all sensory attributes except for taste and flavour. Meanwhile, carrot cake containing 15% concentrated NFS had 5.18 and 5.10 score values of taste and flavour, respectively. Using carrot cake as a bakery product model, this study showed that novel food ingredients like NFS could be used as a natural alternative sweetener up to 20% to replace table sugar to enhance nutritional compositions and antioxidative properties while maintaining the sensory quality of carrot cakes. Even though an improvement was observed in cakes formulated with NFS up to 20%, low values in antioxidant capacity were detected in the present study. Other processing methods such as freeze-drying or higher incorporation of concentrated NFS to replace sugar in cake formulations could be suggested in further research to improve and compare the antioxidant properties of the formulated cake. Besides, further study can be done to investigate the glycaemic response of processed foods incorporated with NFS.

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REFERENCES

Ahmadi, H., Azizi, M. H., Jahanian, L., & Amirkaveei, S. (2011). Evaluation of replacement of date liquid sugar as a replacement for invert syrup in a layer cake. *Iranian Journal of Food Science and Technology*, *8*(1), 57-64.

Aimi, R. N., Bakar, A. F., & Dzulkifly, M. H. (2013). Determination of volatile compounds in fresh and fermented nipa sap (*Nypa fruticans*) using static headspace gas chromatography-mass spectrometry (GC-MS). *International Food Research Journal*, *20*(1), 369-376.

Akochi-K, E., Alli, I., & Kermasha, S. (1997). Characterization of the pyrazines formed during the processing of maple syrup. *Journal of Agricultural and Food Chemistry*, *45*(9), 3368-3373.

Alam, M. N., Bristi, N. J., & Rafiquzzaman, M. (2013). Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143-152.

Ali, H., Houghton, P. J., & Soumyanath, A. (2006). Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *Journal of Ethnopharmacology*, *107*(3), 449-455.

Ancos, B. D., Sgroppo, S., Plaza, L., & Cano, M. P. (2002). Possible nutritional and health-related value promotion in orange juice preserved by high pressure treatment. *Journal of the Science of Food and Agriculture*, *82*(8), 790-796.

Association of Official Analytical Chemists. (2000). *Official methods of analysis* (17th ed.). Rockville, USA: AOAC International.

Association of Official Analytical Chemists. (2012). *Official methods of analysis* (19th ed.). Rockville, USA: AOAC International.

Badarinath, A. V., Mallikarjuna, K., Chetty, C. M. S., Ramkanth, S., Rajan, T. V. S., & Gnanaprakash, K. (2010). A review on *in vitro* antioxidant methods: Comparisions, correlations and considerations. *International Journal of Pharmtech Research*, *2*(2), 1276-1285.

Bhandari, R. M., Jong-Anurakkun, N., Hong, G., & Kawabata, J. (2008). α-glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata*, Haw.). *Food Chemistry*, *106*(1), 247-252.

Brown, A. (2011). *Understanding food principles and preparation* (4th ed.). Boston, USA: Cengage Learning.

Burdulu, H. S., & Karadeniz, F. (2003). Effect of storage on nonenzymatic browning of apple juice concentrates. *Food Chemistry*, *80*(1), 91-97.

Correia, R. T. P., Borges, K. C., Medeiros, M. F., & Genovese, M. I. (2012). Bioactive compounds and phenolic-linked functionality of powdered tropical fruit residues. *Food Science and Technology International*, *18*(6), 539-547.

Fong, F. W. (1992). Perspective for sustainable resource utilization and management of nipa vegetation. *Economic Botany*, *46*(1), 45-54.

Food and Agriculture Organization. (2017). *Food balance sheets*. Retrieved January 6, 2019, from http://www.fao.org/faostat/en/#data/FBS

Food Regulations 1985. (n. d.). Retrieved January 12, 2019, from http://fsis2.moh.gov.my/fosimtestsite/HOM/frmHOMFARSecDetail.aspx?FAS_ID=37&FAC_ID=21&FAD_ID=384

Gibney, M. J., Lanham-New, S. A., Cassidy, A., & Vorster, H. H. (2009). *Introduction to human nutrition* (2nd ed.). San Francisco, USA: Wiley-Blackwell.

Gupta, S., Mahajan, V., Mahajan, S., & Tandon, V. R. (2012). Artificial sweeteners. *Journal of Medical Education and Research*, *14*(1), 1-4.

Hamilton, L. S., & Murphy, D. H. (1988). Use and management of nipa palm (*Nypa fruticans*, Arecaceae): A review. *Economic Botany*, *42*(2), 206-213.

Harpaz, D., Loo, P. Y., Cecchini, F., Koon, T. H. P., Kushmaro, A., Tok, A. I. Y., … Eltzov, E. (2018). Measuring artificial sweeteners toxicity using a bioluminescent bacterial panel. *Molecules*, *23*(10), 2454.

Insel, P., Ross, D., McMahon, K., & Bernstein, M. (2018). *Discovering nutrition*. Burlington, USA: Jones and Bartlett Learning.

Jabatan Pengairan dan Saliran Negeri Kelantan. (2018). *Laporan purata taburan hujan bulanan mengikut tahun di negeri Kelantan* [Monthly average rainfall report by year in Kelantan state]. Retrieved January 6, 2019, from http://www.data.gov.my/data/ms_MY/dataset/laporan-purata-taburan-hujan-bulanan-bagi-tahun-2017

Jaworski, S. (n.d.). *Carrot cake recipe and video*. Retrieved January 13, 2019, from https://www.joyofbaking.com/CarrotCake.html

Kedare, S. B., & Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, *48*(4), 412–422.

Laos, A. K., Kirs, B. E., Kikkas, C. A., & Paalme, D. T. (2007). *Crystallization of the supersaturated sucrose solutions in the presence of fructose, glucose and corn syrup*. Retrieved January 12, 2019, from http://folk.ntnu.no/skoge/prost/proceedings/ecce6_sep07/upload/4066.pdf

Letchuman, G. R., Nazaimoon, W. M. W., Mohamad, W. B. W., Chandran, L. R., Tee, G. H., Jamaiyah, H., … Faudzi, A. Y. (2010). Prevalence of diabetes in the Malaysian National Health Morbidity Survey III 2006. *Medical Journal of Malaysia*, *65*(3), 173-179.

Liu, S. C., Lin, J. T., Wang, C. K., Chen, H. Y., & Yang, D. J. (2009). Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers. *Food Chemistry*, *114*(2), 577–581.

Liyana-Pathirana, C. M., & Shahidi, F. (2006). Importance of insoluble-bound phenolics to antioxidant properties of wheat. *Journal of Agricultural and Food Chemistry*, *54*(4), 1256-1264.

Majzoobi, M., Mansouri, H., Mesbahi, G., Farahnaky, A., & Golmakani, M. T. (2016). Effects of sucrose substitution with date syrup and date liquid sugar on the physicochemical properties of dough and biscuits. *Journal of Agricultural Science and Technology*, *18*(3), 643-656.

Malaysian Meteorological Department. (2019). *Fenomena cuaca: Monsun* [Weather phenomenon: Monsoon]. Retrieved January 6, 2019, from http://www.met.gov.my/pendidikan/cuaca/fenomenacuaca

Manisha, G., Soumya, C., & Indrani, D. (2012). Studies on interaction between stevioside, liquid sorbitol, hydrocolloids and emulsifiers for replacement of sugar in cakes. *Food Hydrocolloids*, *29*(2), 363-373.

Marinovich, M., Galli, C. L., Bosetti, C., Gallus, S., & Vecchia, L. C. (2013). Aspartame, low-calorie sweeteners and disease: Regulatory safety and epidemiological issues. *Food and Chemical Toxicology*, *60*(2013), 109-115.

Matsui, N., Okimori, Y., Takahashi, F., Matsumura, K., & Bamroongrugsa, N. (2014). Nipa (*Nypa fruticans* wurmb) sap collection in Southern Thailand. *Environmental and Natural Resources Research*, *4*(4), 75-88.

Mierlo, L. A. J. V., Greyling, A., & Zock, P. L. (2010). Suboptimal potassium intake and potential impact on population blood pressure. *Journal of American Medical Association Internal Medicine*, *170*(16), 1501-1502.

Minh, N. P. (2014). Various factors influencing to the nipa (*Nypa fruticans*) wine fermentation. *International Journal of Multidisciplinary Research and Development*, *1*(4), 111-114.

Ministry of Health Malaysia. (2017). *Recommended nutrient intakes for Malaysia*. Putrajaya, Malaysia: Author.

Naknean, P., Meenune, M., & Roudaut, G. (2013). Changes in properties of palm sugar syrup produced by an open pan and a vacuum evaporator during storage. *International Food Research Journal*, *20*(5), 2323-2334.

Nguyen, D. V., Rabemanolontsoa, H., & Saka, S. (2016). Sap from various palms as a renewable energy source for bioethanol production. *Chemical Industry and Chemical Engineering Quarterly*, *22*(4), 355-373.

Norimah, A. K., Safiah, M., Jamal, K., Haslinda, S., Zuhaida, H., Rohida, S., … Azmi, M. Y. (2008). Food consumption patterns: Findings from the Malaysian adult nutrition survey (MANS). *Malaysian Journal of Nutrition*, *14*(1), 25-39.

Oyaizu, M. (1986). Studies on products of browning reactions: Antioxidative activities of product of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, *44*(6), 307-315.

Päiväke, A. E. A. (1996). Plant yielding non-seed carbohydrate. In M. Flach & Rumawas (Eds.), *Plant resource of South-East Asia* (pp. 133-137). Bogor, Indonesia: Prosea Foundation.

Shukla, T. P. (1995). Problems in fat-free and sugarless baking. *Cereal Foods World*, *40*(1), 159-160.

Sicree, R., Shaw, J., & Zimmet, P. (2006). The global burden: Diabetes and impaired glucose tolerance - Prevalence and projections. In D. Gan (Ed.), *Diabetes atlas* (pp. 16-103). Brussels, Belgium: International Diabetes Federation.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(48), 144-158.

Stuckel, J. G., & Low, N. H. (1996). The chemical composition of 80 pure maple syrup samples produced in North America. *Food Research International*, *29*(3-4), 373-379.

Sukairi, A. H., Asaruddin, M. R., Sabri, W. M. A. W., & Yusop, S. A. T. W. (2018). Crystallization and biological studies of *Nypa fruticans* Wurmb sap. *Indonesian Journal of Pharmaceutical Science and Technology*, *1*(1), 37-42.

Szawara-Nowak, D., Zielińska, D., Zieliński, H., & Wronkowska, M. (2017). Antioxidant properties of dark wheat bread with exogenous addition of buckwheat flour. In V. Waisundara & N. Shiomi (Eds.), *Superfood and functional food - An overview of their processing and utilization* (pp. 274-289). London, United Kingdom: IntechOpen Limited.

Tamunaida, P., & Saka, S. (2011). Chemical characterization of various parts of nipa palm (*Nypa fruticans*). *Industrial Crops and Products*, *34*(3), 1423-1428.

Trinidad, T. P., Mallillin, A. C., Sagum, R. S., & Encabo, R. R. (2010). Glycemic index of commonly consumed carbohydrate foods in the Philippines. *Journal of Functional Foods*, *2*(4), 271-274.

Uthumporn, U., Woo., W. L., Tajul, A. Y., & Fazilah, A. (2015). Physico-chemical and nutritional evaluation of cookies with different levels of eggplant flour substitution. CyTA-*Journal of Food*, *13*(2), 220-226.

Willits, C. O., & Hills, C. H. (1976). *Maple syrup producers manual, agricultural research service*. Washington, USA: US Department of Agriculture.

World Health Organization. (2013). *WHO issues new guidance on dietary salt and potassium*. Retrieved January 13, 2019, from https://www.who.int/mediacentre/news/notes/2013/salt_potassium_20130131/en/

Yuan, E., Liu, B., Wei, Q., Yang J., Chen, L., & Li, Q. (2014). Structure activity relationships of flavonoids as potent alpha-amylase inhibitors. *Natural Product Communications*, *9*(8), 1173-1176.

Yunos, N. J., Sulaiman, A., Yusuf, N. A., & Ghazali, D. R. (2018). Sugars, minerals composition and metal ion chelating activity of *Arenga pinnata* syrup using vacuum evaporation. *Malaysian Journal of Analytical Sciences*, *22*(2), 264-269.

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The Proximate and Phytochemical Properties of Red Pitaya (*Hylocereus polyrhizus*) Stem Flour and Its Potential Application as Food Products

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ABSTRACT

Red pitaya fruit and peel have been widely explored for food products due to their functional properties. However, the stem still has limited use. This work was aimed to determine the proximate and phytochemical properties of red pitaya stem by processing it as flour and applied it as food products. The different drying temperature (40, 50, 60ºC) on the flouring process was conducted to determine the best drying condition. The best drying temperature was then used to prepare the flour by using whole stem (epidermis and cortex) and peeled stem (cortex only). The result showed that the effective drying temperature on the flouring process was 60ºC. The predominant component in the whole and peeled stem flour was fiber (total of hemicelluloses, cellulose and lignin), which contained up to 50.4% and 43.03% respectively. The second largest component was protein which the whole stem flour contained 9.09% of it and the peeled stem flour contained 11.97% of it. Both of the flour contained high vitamin C (3.64–3.76%) and phenolic compounds (43.55–44.54 mg/g). Either whole or peeled stem flour showed antioxidant activity up to 91% of inhibition and antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* as well as *Salmonella typhi*. The resulting flour has been successfully applied as substitute and additional ingredients to make fiber enriched food products.

Keywords: Drying temperature, phytochemical, pitaya stem flour, proximate analysis

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INTRODUCTION

Red pitaya or known as red dragon fruit (*Hylocereus polyrhizus*) is a member of the Cactaceae family from the Cactoidea subfamily of the Cactea tribe. This fruit has been popular because of its nutritional composition and antioxidant activity. Many researchers have conducted some studies to explore the potential use of red pitaya fruit and peel as prospective functional food products and sources of natural pigment (Ho & Latif, 2016; Jamilah et al., 2011; Rebecca et al., 2010; Tenore et al., 2012). However, the study on utilization of red pitaya stem for food product has not been conducted yet.

Red pitaya stem is an abundant agricultural waste. In the local pitaya plantations in East Java – Indonesia, as much as ±60 ton per hectare, red pitaya stem can be obtained at every harvest time. During that time, red pitaya stem is only limited to be used for transplant and animal feed. Jafaar et al. (2009) reported that the stem of red pitaya still had a high nutritional value, especially the ascorbic acid content which was found to be higher than the fruit flesh. One of the efforts to exploit red pitaya stem is by making it as flour. The flouring process consists of several stages including removal of thorns and bark, reductions of size, drying, flouring, and sieving. The drying temperature is a critical controlled step to produce pitaya stem flour with high vitamin C, since it is easily destroyed in high temperature.

In the other hand, the research on exploration of agricultural biomass as a source of dietary fiber has increased in the last decade (Dungani et al., 2016). Pitaya stem can be a new candidate to be utilized as a source of dietary fiber. The consumption of dietary fiber has been correlated with the prevention of many diseases (Dahl & Stewart, 2015). Thus, determination of proximate and phytochemical properties of red pitaya stem flour is important to be done. In this research, red pitaya stem was processed into flour by using the whole stem (epidermis and cortex) and peeled stem (cortex only). The bark stem (epidermis) of red pitaya is green while the cortex is greenish white. The green color of the plant is generally caused by chlorophyll. Chlorophyll and its derivatives have been reported to have anti-mutagenic activity (Ong et al., 1986) and antioxidants (Lanfer-Marquez et al., 2005). Chlorophyll also contributes to the appearance of the final color of product. In addition to the chlorophyll, bark also has high enough fiber content and vitamin C, so the effect of stripping the bark will have an effect on the nutritional composition and appearance of red pitaya stems flour. The obtained red pitaya stem flours then are analyzed to know its proximate and phytochemical properties. By knowing its characteristics, the flour can be applied to create a new fiber enriched food products.

MATERIALS AND METHODS

Preparation and Production of Red Pitaya Stem Flour

The red pitaya stem used in this research was taken from plantation in Banyuwangi, East Java, Indonesia. The thorns were removed from the stems and then the stems were washed with running water. The whole stem flour was made from both epidermis and cortex of the stem, while peeled stem flour was made only from the cortex of the stem. The whole and peeled stems were thinly sliced (±0.2 mm) then dried in 50ºC using cabinet dryer. The drying process was stopped until it reached a moisture content of ±3-5%. The dried sliced stems were mashed and sieved into 70 mesh size. The obtained flours were then analyzed for proximate and phytochemical analyses and used to make food products hereinafter.

Determination of Drying Temperature for the Production of Red Pitaya Stem Flour

The peeled stem flours dried using three different temperatures (40, 50 and 60ºC) were analyzed for their vitamin C level. The drying time to achieve ±3-5% of moisture content was also monitored. The vitamin C content and drying time were used to determine the best drying temperature. Statistical analysis was conducted using One Way ANOVA (P_{value}<0.05) followed by Tukey multiple comparison test. The selection of drying temperature was based on the shortest time while the vitamin C content still could be preserved. The selected temperature was then used to prepare the red pitaya stem flour for further analyses.

Analysis of Red Pitaya Stem Flour

The whole stem and peeled stem of red pitaya stem flour were analyzed for proximate and phytochemical analyses. All analyses were conducted in triplicate. The difference between samples was determined using T-test (P_{value} <0.05). The protocol for proximate and phytochemical analyses is described as follows.

Proximate Analysis

Moisture Content. As much as 2 g of the sample was placed in the crucible which its constant weight has been known. The sample then was placed inside 105 °C drying oven (Memmert 600, Germany) for 3-5 h and placed in the desiccators afterwards for allowing cooling. The dried sample was weighed until it achieved its constant weight (weighing difference was less than 0.2 mg). The formula for moisture content calculation described as follows:

$$\text{Moisture content (\%)} = \frac{W2 - W3}{W2 - W1} \times 100$$

Note: W1 = weight of crucible; W2 = weight of crucible and sample before drying; and W3 = weight of crucible and sample after drying

Ash. The ash content measurement was conducted by weighing 2 grams of sample and put it into crucible. The total initial weight of the sample and the crucible was recorded. The sample was then placed in a muffle furnace oven (Daihan Scientific FX-14, Korea) at 550°C for 8 h. The sample was placed in the desiccators for cooling and weighed until it achieved its constant weight. The ash content was calculated as:

$$\text{Ash content (\%)} = \frac{W2 - W3}{W2 - W1} \times 100$$

Note: W1 = weight of crucible; W2 = weight of crucible and sample before ashing; and W3 = weight of crucible and sample after ashing

Reducing Sugar and the Total Sugar. The sample was prepared by dissolving the 5 grams of flour into 100 ml distilled water which stirred for 1 hour. The non-soluble part was separated with the soluble part by centrifugation 6,708 × *g* for 10 minutes. The soluble part then taken for further sugar analyses and the added distilled water was calculated as a dilution factor. The concentration of reducing sugar was determined by dinitrosalicylic (DNS) colorimetric assay (Miller, 1959). The total sugar was determined by sulfuric acid phenol method (Dubois et al., 1956). Both of the total and reducing sugar determinations were using glucose as sugar standard, and the formula described as follows:

$$\text{Reducing sugar (\%)} = \frac{\text{Reducing sugar concentration} \times \text{dilution volume}}{\text{Initial weight of sample}} \times 100$$

$$\text{Total sugar (\%)} = \frac{\text{Total sugar concentration} \times \text{dilution volume}}{\text{Initial weight of sample}} \times 100$$

Starch. The concentration of starch was determined by weighing sample of 5 grams and added with 50 ml of distilled water into a 250 ml glass beaker and stirred for 1 hour. The suspension is filtered and washed with distilled water until the volume of filtrate is 250 ml. The filtrate is discarded. The residue on the filter paper was washed 5 times with 10 ml of ether and allowed to evaporate. The residue then washed with 150 ml of 10% alcohol to release dissolved carbohydrates. The residue in the filter paper was then moved to Erlenmeyer and washed with 200 ml of distilled water and 20 ml of 25% HCl. The Erlenmeyer then covered with condenser and then heated in boiling water for 2.5 hours. The solution was allowed to cool then neutralized with 45% NaOH solution and dilution was carried out until the volume was reached 500 ml. The sample then was filtered and the sugar content expressed as glucose was determined from the obtained filtrate by dinitrosalicylic (DNS) colorimetric assay for reducing sugar measurement. Reducing sugar weight was multiplied by 0.9 as the weight of starch. Reducing sugar weight and starch content equations are listed below:

$$\text{Reducing sugar weight} = \text{Reducing sugar concentration} \times \text{dilution volume}$$

$$\text{Starch content (\%)} = \frac{\text{Reducing sugar weight} \times 0.9}{\text{Initial weight of sample}} \times 100$$

Lignocelluloses. The analysis of lignocelluloses (lignin, cellulose, and hemicelluloses) was conducted according to Chesson (1981). As much as one gram of flour sample (a) and 150 ml of distilled water were mixed and heated in a 95ºC water bath for 1 hour. The mixture was filtrated and the residue was rinsed with 300 ml of hot water. The residue was dried in an oven to a constant weight (b). The residue was added with 150 ml of H_2SO_4 1N and heated in a 90-100ºC water bath for an hour. The mixture was filtered and rinsed with 300 ml of hot water. Then, the residue was dried (c). Dry residue was soaked in 10 ml of 72% H_2SO_4 for 4 hours at room temperature. After that, 150 ml of H_2SO_4 1N was added to the mixture and refluxed in the water bath for 1 hour. The solid was rinsed with 400 ml of distilled water and heated in an oven at 105ºC and a constant weight was weighed. The solid was burnt in furnace and the ash was weighed (e). The formula for calculation of percent cellulose, hemicellulose, and lignin described as follows:

$$\text{Hemicellulose (\%)} = \frac{b - c}{a} \times 100$$

$$\text{Cellulose (\%)} = \frac{c - d}{a} \times 100$$

$$\text{Lignin (\%)} = \frac{d - e}{a} \times 100$$

Note: a = weight of sample (gram); b = weight of first residue (gram); c = weight of second residue (gram); d = weight of third residue (gram); e = weight of ash (gram)

Crude Fiber. As much as 2 grams of fat-free sample was added with 50 ml of 1.25% H_2SO_4 and put into 500 ml Erlenmeyer. This mixture was boiled for 30 minutes using condenser, and was added with 3.25% NaOH then boiled again for 30 minutes. The samples then immediately filtered in hot condition using a Buchner funnel with non-gray Whatman 54, 41, or 541 filter paper which had been dried and known for its weight. The residue on the filter paper was washed sequentially with 1.25% hot H_2SO_4, hot water, and 96% ethanol. The filter paper and its contents were lifted and placed on a crucible (that has known for its weight) and dried at 105ºC. The sample was then cooled and guided until a fixed weight was obtained. The weight of the residue represents the weight of crude fiber. The percent of the crude fiber was calculated using the following formula:

$$\text{Crude fiber (\%)} = \frac{\text{Weight of crude fiber}}{\text{Weight of sample}} \times 100$$

Crude Protein. The crude protein content was evaluated using micro-Khjeldal method (Koch & McMeekin, 1924). As much as 1 g of the sample was placed inside a Kjeldahl flask. The sample was then added with 15 g of K_2SO_4, 1 mg of $CuSO_4$ catalyst solution, 1 g of catalyst selen, boiling stone and 25 mg of concentrated H_2SO_4. The mixture then was boiled and the color changed into a clear green. The mixture was then cooled off and diluted using distilled water as needed. 75 ml of 30 % NaOH solution was given before it was distilled for 5–10 min until the solution reached 150 mL, with 50 mL of 4 % H_3BO_3 solution posing as the container. The solution was then titrated using 0.1 N HCl. The difference in the total value of the titrated sample and the blank, posed as the total value of nitrogen. The protein content is obtained through the process of multiplying N% with 6.25 convection factor.

$$N (\%) = \frac{(\text{mL of HCl in sample} - \text{mL of HCl in blank}) \times \text{N HCl} \times 1.4007}{\text{Weight of sample in gram}} \times 100$$

$$\text{Protein content} (\%) = \text{N} \% \times 6.25$$

Fat. The fat content was measured by directly extracting the pitaya stem flour with petroleum ether in Soxhlet extractor for 4 h. The residue after solvent removal in round bottom flask represents the fat content of the sample. The fat content calculation was conducted as follows:

$$\text{Fat content} (\%) = \frac{\text{Weight of the residue}}{\text{Weight of sample}} \times 100$$

Phytochemical Analysis

Vitamin C. Vitamin C was measured by mixing 100 mg of sample with 10 ml of distilled water and then stirred for 2-3 minutes. The solution was added with 5 ml of starch indicator and titrated with 0.1 N Iodine solution which was standardized with $Na_2S_2O_3$ solution which has standardized by KIO_3 0.1 N as the primary standard solution. Then, the titration was stopped when the solution titrated with iodine solution was dark blue and lasted for about 1 minute.

$$\text{Vitamin C} (\%) = \frac{\text{Mr of Ascorbic Acid} \times \text{N iodine} \times \text{V iodine (L)}}{\text{Weight of the sample}} \times 100$$

Antioxidant Activity. Testing of antioxidant activity was carried out using DPPH reagent (2, 2-diphenyl-1-picrylhydrazyl). The test was carried out by adding 0.5 ml 10,000 ppm of the sample with 0.5 ml of DPPH 0.1 mM (in ethanol). The control solutions were made by replacing samples with sample solvents and added with 0.5 ml of DPPH 0.1 mM (in ethanol). Blank solutions were made by using sample solvents, while all tubes were incubated in a dark room for 30 minutes. After that, measurements were taken by reading the absorbance using a spectrophotometer at a wavelength of 517 nm (Marques et al., 2012). The percent of inhibition was calculated using this formula:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - (\text{absorbance of sample} - \text{blank})}{\text{Absorbance of control}} \times 100$$

Total Phenolic Compound. As much as 200 µl of 2,000 ppm flour samples in water were added with 2.5 ml of Folin-Ciocalteau reagent (10% v/v) and 2 ml of Na_2CO_3 (7.5% w/v) and homogenized then incubated for 15 minutes at 45 ℃. The absorbance of the solution was then measured by a spectrophotometer at a wavelength of 765 nm. The total phenolic compounds expressed as milligrams (mg) gallic acid equivalent per gram of sample (mg GAE/g sample). As a standard, gallic acid was used in ethanol at various concentrations (0, 5, 10, 20, 40, 80 and 100 ppm) (Javanmardi et al., 2003).

Antibacterial Activity. A total of 100 mg of red pitaya stem (whole and peeled) flour samples were dissolved into 100 µl distilled water to be tested for antimicrobial activity. Antimicrobial activity was aimed primarily towards pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*. Each of these pathogenic microbes was grown in nutrient broth to reach $OD_{600} = 0.5$. Furthermore, antimicrobial testing using the pour plate technique was carried out by adding 100 µL of bacteria to 15 ml of nutrient agar and homogenized. The media was then poured into sterile petri dishes and left until the media was solidified. Sterile cylinder cups were planted partially on the media to contain microbial inoculums and added with 100 µl of positive control, negative control, and samples. The media then was incubated at 37ºC for 24 hours. Observations were made by determining the diameter of the clear zone formed around the cylinder cup. The positive controls used for *Staphylococcus aureus* and *Escherichia coli* were ampicillin (100 mg/ml) and chloramphenicol (5 mg/ml) respectively, while the negative controls used were distilled water as the solvents of the samples.

Toxicity Test. Toxicity test was carried out using the Brine Shrimp Lethality Test (BSLT) method using *Artemia salina*. As much as 10 mg of shrimp eggs *A. salina* was grown in 100 ml of filter-sterilized seawater. The hatching temperature was ± 25-30ºC and pH ± 6-7 for 48 h. After the hatching process, the active nauplii were collected and used for the assay. As much as 20 ml of pitaya stem flour in brine solution (250 ppm, 500 ppm, 1000 ppm and 1500 ppm) was put into a petri dish containing 20 nauplii and incubated at room temperature for 24 h and surviving larvae were counted. The experiments were conducted along with control and each treatments were conducted in triplicate.

The Application of Red Pitaya Stem Flour on Food Products

Red pitaya whole stem and peeled stem flour were applied to enrich several food products, i.e. cake, cookies, noodle, pudding, yoghurt and jelly drink. Descriptive sensory evaluations were carried out for those products using five selected trained panelist. The selection began with thirty people from both students and staffs in our institution who committed to attend the training and evaluation sessions. The screening procedure to obtain five selected trained panelist was based on Meilgaard et al. (1999). The trained panelists were involved to generate lexicon to evaluate several food products enriched with pitaya stem flour which consist of appearance, aroma, taste, color and texture attributes using a 0–15 scale. The food products composition were added or substituted with pitaya stem flour using following proportions: yoghurt (addition of 1, 2 and 3%), cake (20% substitution of total flour), cookies (25% substitution of total flour), noodle (substitution 5, 10 and 15% of total flour), pudding (addition of 1, 2 and 3%) and jelly drink (addition of 1, 2 and 3%). Analysis of variance (ANOVA) followed with Tukey test were used to determine the differences between samples, excepting cake and cookies data analysis were using one tailed T-test. All statistical analyses were performed using IBM SPSS Statistic 24 (SPSS Inc, USA).

RESULTS AND DISCUSSION

The effect of drying temperature on characteristics of peeled red pitaya stem flour is listed in Table 1. Vitamin C content is an important parameter to be monitored during drying process because vitamin C is thermally unstable. In the other hand, vitamin C is an important nutrient components which has function as antioxidants and prevent various diseases (Chen et al., 2013). The results showed that vitamin C levels of red pitaya stem flour were affected by drying temperatures, where there was a decreasing in vitamin C levels with the increasing of drying temperature. Statistically, there is a significant difference in the level of vitamin C in the treatment between 40 and 50 or 60°C. However, there is no significant difference between 50 and 60°C. Another study conducted by El-Ishaq and Obirinakem (2015) was in line with this study, where vitamin C levels were lower due to high temperature treatment of fruit juice. Vitamin C is easily oxidized when it is in contact with air or light at high temperatures.

The drying time to achieve ±3-5% moisture of red pitaya stem flour was monitored. The data is listed in Table 1. The result shows that drying time was affected

Table 1
The effect of drying temperature on characteristics of peeled red pitaya stem flour

Parameter	Temperature (°C)		
	40	50	60
Vitamin C (%)	$4.23^a \pm 0{,}24$	$3.73^b \pm 0{,}10$	$3.64^b \pm 0.13$
Moisture (%)	$5.13^a \pm 1{,}29$	$4.88^a \pm 1{,}69$	$3.61^a \pm 0.53$
Drying time (h)	$48.43^a \pm 1{,}45$	$37.28^b \pm 2.45$	$19.95^c \pm 1.54$

Note: Different letter notations behind the mean in each row indicates a significant difference based on Tukey test (P-value <0.05) by drying temperature, where the higher drying temperature the shorter drying time. Drying temperature of 60°C was selected for further drying process because it took the shortest time but still maintained the vitamin C level which was not significantly different compared to 50°C. The color of peeled red pitaya stem flour under different drying temperatures is shown on Figure 1. The increasing drying temperature made the flour color changed from light green to light yellowish green. The green color was contributed by chlorophyll, a natural pigment present in a plant. Chlorophyll is less stable in high temperature. As reported by Van Loey et al. (1998), degradation of chlorophyll occurred when it was processed in high temperature.

The selected drying temperature then was applied to proceed for both whole stem and peeled stem of red pitaya to become flour. The obtained flour was analyzed and the results listed in Table 2. The moisture content was maintained at the same level for the two samples. There was a significant difference on protein and starch content between the whole and peeled stem flour. The high protein and starch in peeled stem flour is due to the cortical cells may contain stored carbohydrates or other substances such as resins, latex, essential oils, and tannins (Glimn-Lacy & Kaufman, 2012). There was also a significant difference on fat content between whole and peeled stem flour. The high level of fat in whole stem flour is due to the presence of wax in the epidermis of the stem. The stems of plant

Table 2
Proximate analysis of red pitaya stem flour

Parameter	Whole stem flour	Peeled stem flour
Moisture (%)	$3.42^a \pm 0.88$	$3.61^a \pm 0.53$
Protein (%)	$9.09^b \pm 0.52$	$11.97^a \pm 0.87$
Fat (%)	$0.89^a \pm 0.26$	$0.12^b \pm 0.16$
Starch (%)	$0.57^b \pm 0.13$	$1.91^a \pm 0.43$
Hemicelluloses (%)	$15.40^b \pm 0.50$	$27.42^a \pm 1.60$
Cellulose (%)	$32.59^a \pm 0.75$	$13.14^b \pm 1.76$
Lignin (%)	$2.46^a \pm 0.45$	$2.47^a \pm 0.40$
Crude Fiber (%)	$24.48^a \pm 3.19$	$14.65^b \pm 2.42$
Ash (%)	$1.69^a \pm 0.56$	$2.17^a \pm 0.69$
Total Sugar (%)	$5.07^a \pm 0.79$	$6.56^a \pm 0.91$
Reducing Sugar (%)	$1.67^a \pm 0.63$	$2.34^a \pm 0.82$

Note: Different letter notations behind the mean in each row indicates a significant difference based on T-test with a P-value of 0.05

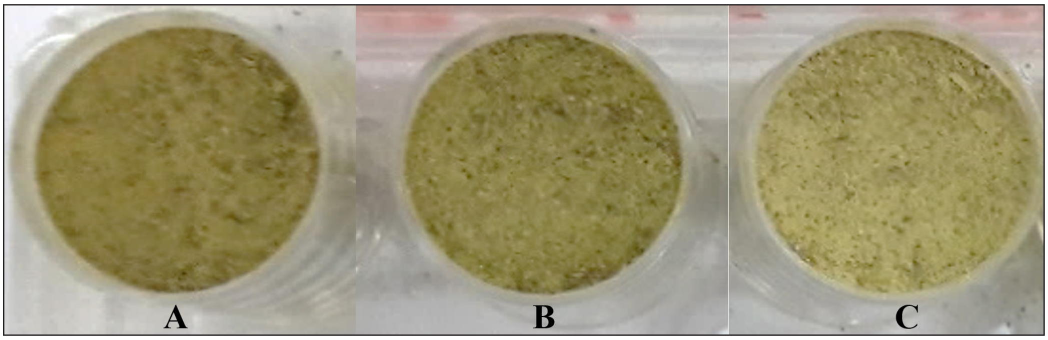

Figure 1. Comparison of peeled red pitaya stem flour's colour on different drying temperature. (A) 40°C; (B) 50°C; (C) 60°C

sometimes covered with smooth layers of wax which give them a whitish surface color and protect them from intense sunlight by acting as a moisture barrier (Raven, et al., 1981). From the data listed in Table 2, it also can be seen that there is a significant difference on crude fiber, hemicellulose and cellulose content between whole and peeled stem flour. Hemicellulose is a polysaccharide matrix found in plant biomass about 20-30% of the plants dry weight. It is stated that hemicellulose is a polysaccharide matrix as a filler of space between cellulose along with lignin in plant cell walls (Bergander & Salmen, 2002). However, there is no significant difference between whole and peeled stem flour on lignin, ash, starch, total sugar and reducing sugar content. Jaafar et al. (2009) suggested that red pitaya stems had a total ash content of 1.5% in young stems and 4.5% in stems that were quite old.

Phytochemical properties of red pitaya stem flour were shown in Table 3. Statistically, there was no significant difference of vitamin C and antioxidant activity for whole stem and peeled stem flour. However, there was a significant difference on the total phenolic compound between whole stem and peeled stem flour. It indicates that the epidermis contributes to higher phenolic compound than the cortex. The differential accumulation of the total phenols is associated with differential cytological and physiological activities within tissue and organs. The production of these compounds is highly ordered process and regulated by differential expression of genes involved in phenylpropanoid pathway (Chang et al., 2009; Mamti et al., 2006). The phenolic compounds also reported to have antioxidant activity against free radical compounds (Loganayaki et al., 2013). However, in this experiment there was no clear relationship between the levels of phenolic compounds and antioxidant activity. Where the total of phenolic compounds between whole and peeled flour differed significantly, the antioxidant activity was not significantly differed. This can be caused by differences in phenolic components in the epidermis of pitaya stem which can cause different antioxidant activities, depending on the phenolic structure (Nićiforović et al., 2010). This result is also in accordance with other findings reported by Bajpai et al. (2005) and Sengul et al. (2009) which stated no correlation between total phenolic content and antioxidant activities of medicinal plant

Table 3
Phytochemical properties of red pitaya stem flour

Parameter	Whole stem flour	Peeled stem flour
Vitamin C (%)	$3.76^{a} \pm 0.18$	$3.64^{a} \pm 0.13$
Total Phenolic Compound (mg/g)	$44.54^{a} \pm 0.11$	$43.55^{b} \pm 0.19$
Antioxidant Activity (% inhibition)*	$90.89^{a} \pm 2.77$	$90.67^{a} \pm 0.86$

Note: Different letter notations behind the mean in each row indicate a significant difference based on T-test with a P-value of 0.05. *Ascorbic acid was used as positive control for antioxidant activity assay. The percent of inhibition of ascorbic acid was 91.46%

extracts. Moreover, the observed antioxidant activity was not only from the phenolic compounds, but also from the presence of other phytochemicals such pigments and vitamins as well as the synergistic effects among them. On the other hand, Folin Ciocalteu method used for total phenolic content determination is not an absolute measurement of the amount of phenolic substances (Sengul et al., 2009).

From antibacterial activity test (Table 4), it is now known that both whole and peeled red pitaya stem flour provide antimicrobial activity against *E. coli*, *S. aureus* and *S. typhi* because they showed significant difference inhibitory size compared to negative controls. Phytochemical compounds such as tannins, flavonoids, alkaloids and several other aromatic compounds which are secondary metabolites of plants, play a role in defense mechanisms to fight predators such as microorganisms, insects, and herbivores (Doughari, 2006).

The toxicity test of pitaya stem flour samples was carried out by the BSLT method using *Artemia salina* L. larvae. BSLT usually used to determine the cytotoxicity and effectiveness of traditional medicines derived from plants because this method is very easy, inexpensive, and harmless. The procedure determines LC_{50} of active compounds and extracts in the brine medium. Activities of a broad range active compounds are manifested as toxicity to the shrimp (Meyer et al., 1982). In this present study, BSLT test was conducted to reveal the toxicity possibility of the pitaya flour. Toxicity test was determined based on the percent of lethality *A. salina* at various concentrations of pitaya flour as shown in Table 5. At the concentrations of 250 and 500 ppm both whole and peeled stem flour give 0% mortality. While at the concentrations of 1,000 and 1,500 ppm whole stem flour, the mortality percentage was 6.67% and 16.67% respectively. At the concentrations of 1,000 and 1,500 ppm of peeled stem flour, the mortality percentage was 3.33% and 13.33% respectively. Based on Friedman test analysis at the concentrations of 1,000 and 1,500 ppm (p-value of 0.519), showed that there was no significant difference in mortality percentage between samples. This indicates that there was no significant

Table 4
Antibacterial activity of red pitaya stem flour

Sample	Inhibitory size (mm)		
	Escherichia coli	*Staphylococcus aureus*	*Salmonella typhi*
Positive Control	21,47 ± 0,35	21,29 ± 0,03	31,82 ± 0,77
Peeled Stem Flour	$8{,}50^{a}$ ± 0,22	$8{,}54^{a}$ ± 0,19	$8{,}94^{a}$ ± 0,15
Whole Stem Flour	$8{,}44^{a}$ ± 0,11	$8{,}86^{a}$ ± 0,27	$9{,}03^{b}$ ± 0,11
Negative Control	$7{,}95^{b}$ ± 0,23	$7{,}53^{b}$ ± 0,16	$7{,}56^{c}$ ± 0,04

Note: Positive control for *Escherichia coli* and *Staphylococcus aureus* is Ampicillin (100 mg / ml), while positive control for *Salmonella typhi* is Chloramphenicol (5 mg/ml), negative control is distillated water. The letters behind the numbers in one column show a significant difference of treatments and negative control based on Tukey test with a P-value of 0.05

Table 5
Toxicity test based on mortality percentage of Artemia salina *L.*

Flour concentration (ppm)	Mortality percentage of *Artemia salina* L. (%)	
	Peeled stem flour	Whole stem flour
0	0	0
250	0	0
500	0	0
1,000	3.33[a] ± 5.77	6.67 [a] ± 5.77
1,500	13.33[a] ± 5.77	16.67[a] ± 5.77

Note: The same letter notations behind the mean indicate no significant difference based on Nonparametric Test with a P-value of 0.05

difference of possible toxic effect in both flours in those concentrations. Compared to 250-500 ppm, the higher concentration of the flour increased the percentage of nauplii's mortality. Pitaya flour addition has increased the viscosity of the medium due to its high fiber content, causing an increase of osmotic pressure, disrupting nauplii movement and driving the nauplii to death. The addition of the flour higher than 1,500 ppm to growth medium for BSLT assay was not possible to be done due to viscous effect caused. However, the determination of LC_{50} could not be conducted yet. On the other hand, it is possible that phytochemical compounds contains in the flour may cause the death of *Artemia salina* L. larvae. From this data, it still cannot be concluded yet whether the toxicity is caused by viscosity or the presence of phytochemical compounds. It is suggested that further BSLT assay using phytochemical extract of the flour instead of using the whole flour should be conducted. Compared to the other medicinal plants which mostly showed LC_{50} value at concentration below 1,000 ppm (Krishnaraju, et. al., 2005; Madjos & Luceño, 2019), this flour was assumed to be less toxic.

The addition of pitaya stem flour on several food products and their descriptive sensory evaluation are listed in Table 6. In yoghurt, pudding and jelly drink, the trained panelists were revealed ten specific attributes. Most of those attributes were found to be significantly different ($p<0.05$) among of yoghurt, pudding and jelly drink samples, except for smoothness and sourness in yoghurt. The higher concentrations of pitaya stem flour in those three products, the consistency of the products become more viscous. The increase of viscosity of the product may be due to the presence of soluble fiber in pitaya stem flour. Soluble fibers thicken when mixed with fluids and have been reported to have beneficial physiological effects in human, animal, and in vitro models (Dikeman & Fahey Jr., 2006).The bitterness in those three food products were also found to be significantly different ($p<0.05$), whereas bitterness might be caused by phytochemical compounds presence in pitaya stem flour. The addition of pitaya stem flour also affected the colour

Table 6
Descriptive sensory analysis of food products enriched with pitaya stem flour

Attributes		Yoghurt Pitaya flour addition (%)			
		0	1	2	3
Appearance	Smoothness	8.12ᵃ	7.63ᵇ	6.91ᵇ	6.32ᵇ
	Greenish colour	1,61ᵈ	3,83ᶜ	6,34ᵇ	8,1ᵃ
Texture in mouth	Thickness	5.61ᵃ	5.72ᵃ	5.64ᵃ	5.9ᵃ
	Graininess	1,1ᵇ	2.4ᵃ	2.78ᵃ	3.01ᵃ
Taste	Sourness	3.03ᵃ	3.21ᵃ	3.44ᵃ	3.61ᵃ
	Astringent	1.82ᵇ	2.23ᵇ	3.41ᵃ	4.43ᵃ
	Bitterness	2.21ᵈ	3.62ᶜ	5.42ᵇ	7.31ᵃ
	Green tea like	2.01ᵈ	5.63ᶜ	8.22ᵇ	12.4ᵃ
Attributes		**Pudding Pitaya flour addition (%)**			
		0	**1**	**2**	**3**
Appearance	Syneresis	8.12ᵃ	6.56ᵇ	5.02ᶜ	3.87ᵈ
	Smoothness	12.87ᵃ	11.54ᵇ	9.67ᶜ	8.03ᶜ
	Greenish colour	1.56ᶜ	5.6ᶜ	9.94ᵇ	13.9ᵃ
Texture in mouth	Thickness	8.06ᵇ	9.65ᵇ	11.01ᵃ	11.98ᵃ
	Smoothness	12.1ᵃ	11.98ᵃ	11.87ᵃ	10.56ᵇ
Taste	Sweetness	13.03ᵃ	11.21ᵇ	10.44ᵇ	9.61ᶜ
	Astringent	1.82ᶜ	2.21ᶜ	3.47ᵇ	4.49ᵃ
	Bitterness	2.21ᵈ	5.62ᶜ	7.42ᵇ	10.31ᵃ
	Green tea like	2.01ᵈ	5.63ᶜ	8.78ᵇ	12.48ᵃ
Attributes		**Jelly drink Pitaya flavor addition (%)**			
		0	**1**	**2**	**3**
Appearance	Smoothness	8.12ᵃ	8.02ᵃ	7.72ᵇ	7.01ᵇ
	Greenish colour	2.01ᵈ	4.56ᶜ	8.04ᵇ	12.34ᵃ
Texture in mouth	Thickness	4.06ᶜ	5.65ᵇ	6.01ᵇ	7.98ᵃ
Taste	Sweetness	13.83ᵃ	11.61ᵇ	10.74ᵇ	9.81ᵇ
	Astringent	2.02ᵈ	6.21ᶜ	8.47ᵇ	10.49ᵃ
	Bitterness	2.21ᵈ	6.62ᶜ	8.42ᵇ	10.31ᵃ
	Green tea like	2.01ᵈ	6.63ᶜ	8.78ᵇ	12.48ᵃ
Attributes		**Cake Pitaya flour substitution**			
		0		**20**	
Appearance	Puffiness	12.4ᵃ		10.04ᵇ	
	Uniformity	14.1ᵃ		11.3ᵇ	
	Greenish colour	3.8ᵇ		10.87ᵃ	
Texture	Puffiness	12.4ᵃ		8.7ᵇ	
	Crumbliness	3.53ᵃ		2.83ᵃ	

Table 6 *(continue)*

Attributes		Cake Pitaya flour substitution 0	20
Texture	Softness	13.5 a	12.3 b
Taste	Sweetness	11.3 a	10.2 b
	Green tea like	2.1 b	12.2 a
Aroma	Bitterness	1.8 b	4.23 a
	Green tea like	2.1 b	12.2 a
	Baked	14.52 a	14.1a
	Grassy like	2.2 b	11.4 a

Attributes		Noodle Pitaya flour substitution (%) 0	5	10	15
Appearance	Smoothness	11.3 a	11.43 a	11.01 a	10.97 a
	Firmness	12,41d	11.8 c	11.01 b	8,1 a
	Greenish colour	2.03 d	3.03 c	5.21 b	10.03 a
Texture	Elasticity	13.01 a	12.4 a	11.8 a	9.6 b
	Softness	11,1 a	11.4 a	10.08 a	9.7a
Taste	Starchy like	3.03 a	3.21 a	3.44 a	3.61 a
	Grassy like	1.82 b	2.23 b	3.41 a	4.43 a
	Green tea like	2.21d	3.62 c	5.44 b	7.31 a
Aroma	Bitterness	1.1 b	1.5 b	1.7 b	1.86 a
	Grassy like	1.3 a	1.7 a	1.9 a	2.01 a
	Green tea like	1.5c	2.4 b	3.14 b	4.51 a

Attributes		Cookies Pitaya flour substation (%) 0	25
Appearance	Baked colour	12.1 a	10.5 b
	Uniformity	13.1 a	13.1 a
	Greenish colour	2.6 b	4.67 a
Texture	Hardness	6.63 a	6.63 a
	Crunchiness	10.03 a	9.1a
	Softness	9.1 a	9.1 a
Taste	Sweetness	12.01 a	12.01 a
	Green tea like	2.6 b	9.2 a
	Bitterness	3.3 b	6.23 a
Aroma	Green tea like	3.3 b	8.65 a
	Baked	12.4 a	10.3 b
	Grassy like	2.1 b	5.3 a

Note: The scale ranging from 0 – 15 (low to high). Different letter notations behind the mean in each row indicate a significant difference based on Tukey test (P-value <0.05) and T-test (P-value <0.05) (only for cake and cookies data analysis) of the three food products. The more pitaya stem flour added, the colour of the yoghurt, pudding and jelly drink became more greenish (Figures 2D, 2E and 2F).

The attributes assessment in cake, cookies, and noodle were listed in twelve specific attributes. The attributes of texture (puffiness and softness) in cake among the tested samples had significantly different ($p<0.05$). Substitution of wheat flour in the cake making also made the total gluten content in the dough reduced. Gluten is a protein in wheat flour and plays an important role in cake baking performance, which contributes to the rise ability of the dough and maintain the cake's shape when it is baked (Khatkar et al., 1995). The high fiber content in pitaya stem flour causes an increase in the water binding capacity that makes texture of the cake becomes less puff

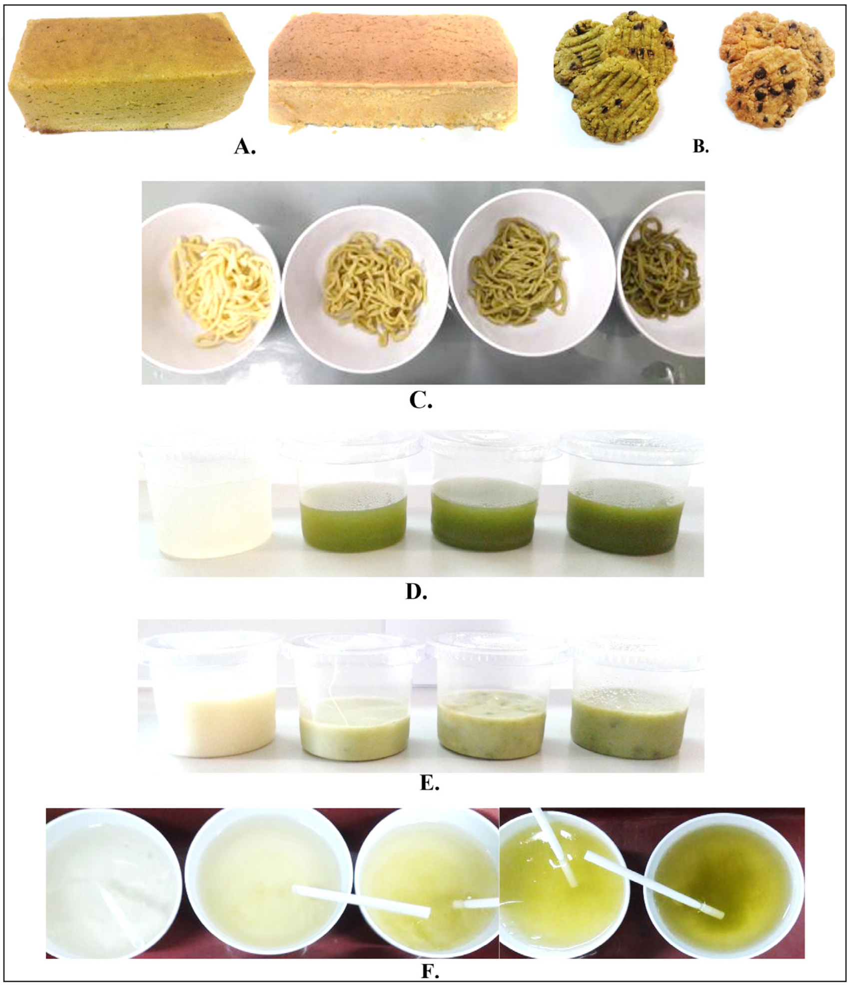

Figure 2. Various food products enriched with pitaya stem flour. A. Cake (Left: 20 % substitution, Right: control); B. Cookies (Left: 25 % substitution, Right: control); C. Noodle (left to right: 0, 5, 10, 15 % substitution); D. Pudding (left to right: 0, 1, 2, 3 % addition); E. Yoghurt (left to right: 0, 1, 2, 3 % addition); F. Jelly drink (left to right: 0, 1, 2, 3, 4 % addition)

and soft (Yamazaki et al., 2005). Therefore, it is recommended to apply pitaya stem flour on cake products lower than 20%. In addition, substitution of wheat flour with pitaya stem flour made the colour of the cake more greenish (Figure 2A). In pitaya flour addition ranging from 5-15% were found to have no significantly different ($p>0.05$) among most of attributes in noodle samples, however elasticity, firmness, and greenish color given significantly different ($p<0.05$). The higher pitaya stem flour addition makes the color become more greenish (Figure 2C) and the texture less elastic. The elasticity is caused by gluten contained in wheat flour (Shewry et al., 1995). Substitution of wheat flour with pitaya stem flour reduced the gluten in the noodle so that the texture becomes less elastic. The appearance (baked and greenish color), taste (bitterness), and aroma attributes of cookies were found to be significantly different ($p<0.05$). The color of pitaya stem cookies represented in Figure 2B. However, the texture assessment in the cookies were found to have no significantly different ($p>0.05$) between the samples.

CONCLUSION

The red pitaya stem flour can be prepared using 60°C drying temperature. This temperature can reduce the moisture content efficiently while the vitamin C content still preserved. The predominant component in whole stem flour was cellulose, followed by hemicellulose and protein. While the predominant component in peeled stem flour was hemicelluloses, followed by cellulose and protein. Both whole and peeled pitaya stem flour can be used as functional food because it contains high vitamin C and phenolic compounds. Moreover it also shows antioxidant activity and antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* as well as *Salmonella typhi*. The resulting flour has been successfully applied as substitute and additional ingredients to make fiber enriched food products.

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REFERENCES

Bajpai, M., Pande, A., Tewari, S. K., & Prakash, D. (2005). Phenolic contents and antioxidant activity of some food and medicinal plants. *International Journal of Food Sciences and Nutrition*, *56*(4), 287–291. doi: 10.1080/09637480500146606

Bergander, A., & Salmén, L. (2002). Cell wall properties and their effects on the mechanical properties of fibers. *Journal of Materials Science*, *37*(1), 151–156.

Chang, J., Lu, J., & He, G. (2009). Regulation of polyphenols accumulation by combined expression silencing key enzymes of phenylpropanoid pathway. *Acta Biochimica et Biophysica Sinica*, *41*(2), 123–130.

Chen, G. C., Lu, D. B., Pang, Z., & Liu, Q. F. (2013). Vitamin C intake, circulating vitamin C and risk of stroke: A meta-analysis of prospective studies. *Journal of the American Heart Association*, *2*(6), e000329.

Chesson, A. (1981). Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. *Journal of the Science of Food and Agriculture*, *32*(8), 745–758.

Dahl, W. J., & Stewart, M. L. (2015). Position of the academy of nutrition and dietetics: Health implications of dietary fiber. *Journal of the Academy of Nutrition and Dietetics*, *115*(11),1861–1870.

Dikeman, C. L., & Fahey Jr., G. C. (2006). Viscosity as related to dietary fiber: A review. *Critical Reviews in Food Science and Nutrition*, *46*(8), 649–663.

Doughari, J. H. (2006). Antimicrobial activity of *Tamarindus indica* Linn. *Tropical Journal of Pharmaceutical Research*, *5*(2), 597–603.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Dungani, R., Karina, M., Subyakto, Sulaeman, A., Hermawan, D., & Hadiyane A. (2016). Agricultural waste fibers towards sustainability and advanced utilization: A review. *Asian Journal of Plant Sciences*, *15*(1–2), 42–55.

El-Ishaq, A., & Obirinakem, S. (2015). Effect of temperature and storage on vitamin C content in fruits juice. *International Journal of Chemical Sciences*, *1*(2), 17–21.

Glimn-Lacy, J., & Kaufman, P. B. (2012). *Botany illustrated: Introduction to plants, major groups, flowering plant families*. New York, NY: Springer Publishing.

Ho, L. H., & Latif, N. W. A. (2016). Nutritional composition, physical properties, and sensory evaluation of cookies prepared from wheat flour and pitaya (*Hylocereus undatus*) peel flour blends. *Cogent Food and Agriculture*, *2*(1), 11363–11369.

Jaafar, R. A., Rahman, A. R. B. A., Mahmod, N. Z. C., & Vasudevan, R. (2009). Proximate analysis of dragon fruit (*Hylocereus polyrhizus*). *American Journal of Applied Sciences*, *6*(7), 1341–1346.

Jamilah, B. Shu, C. E., Kharidah, M., Dzulkily, M. A., & Noranizan, A. (2011). Physico-chemical characteristics of red pitaya (*Hylocereus polyrhizus*) peel. *International Food Research Journal*, *18*(1), 279–286.

Javanmardi, J., Stushnoff, C., Locke E., & Vivanco, J. M. (2003). Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. *Journal of Food Chemistry*, *83*(4), 574–550.

Khatkar, B. S., Bell, A. E., & Schofield, J. D. (1995). The dynamic rheological properties of glutens and gluten sub-fractions from wheats of good and poor bread making quality. *Journal of Cereal Science*, *22*(1), 29–44. doi: 10.1016/S0733-5210(05)80005-0

Koch, F. C., & McMeekin, T. L. (1924). A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. *Journal of the American Chemical Society*, *46*(9), 2066–2069.

Krishnaraju, A. V., Rao, T. V. N., Sundararaju, D., Vanisree, M., Tsay, H. S., & Subbaraju, G. V. (2005). Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *International Journal of Applied Science and Engineering*, *3*(2), 125-134.

Lanfer-Marquez, U. M., Barros, R. M. C., & Sinnecker, P. (2005). Antioxidant activity of chlorophylls and their derivatives. *Food Research International*, *38*(8–9), 885-891.

Loganayaki, N., Siddhuraju, P., & Manian, S. (2013). Antioxidant activity and free radical scavenging capacity of phenolic extracts from *Helicteres isora* L. and *Ceiba pentandra* L. *Journal of Food Science and Technology*, *50*(4), 687–695. doi: 10.1007/s13197-011-0389-x

Madjos, G. G., & Luceño, A. M. (2019). Comparative cytotoxic properties of two varieties of *Carica* papaya leaf extracts from Mindanao, Philippines using Brine Shrimp Lethality Assay. *Bulletin of Environment, Pharmacology and Life Sciences*, *8*(2), 113-118.

Mamti, G. E., Liang, Y., & Lu, J. (2006). Expression of basic genes involved in tea polyphenol synthesis in relation to accumulation of catechins and total tea polyphenols. *Journal of the Science of Food and Agriculture*, *86*(3), 459–464.

Marques, M. R., Paz, D. D., Batista, L. P. R., Barbosa, C. O., Araújo, M. A. M., & Moreira-Araújo, R. S. R. (2012). An *in vitro* analysis of the total phenolic content, antioxidant power, physical, physicochemical, and chemical composition of *Terminalia catappa* Linn. fruits. *Ciência e Tecnologia de Alimentos*, *32*(1), 209–213.

Meilgaard, M., Civille, G. V., & Carr, B. T. (1999). *Sensory evaluation techniques* (3rd ed.). Boca Raton, USA: CRC Press.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobson, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, *45*(5), 31–34.

Miller, G. L. (1959). Use of DNS reagent for determination of reducing sugars. *Analytical Chemistry*, *31*(3), 426–428. doi: 10.1021/ac60147a030

Nićiforović, N., Mihailović, V., Mašković, P., Solujić, S., Stojković, A., & Muratspahić, D. P. (2010). Antioxidant activity of selected plant species; potential new sources of natural antioxidants. *Food Chemistry and Toxicology*, *48*(11), 3125–3130. doi: 10.1016/j.fct.2010.08.007

Ong, T., Whong, W. Z., Stewart, J., & Brockman, H. E. (1986). Chlorophyllin: A potent antimutagen against environmental and dietary complex mixtures. *Mutation Research Letters*, *173*(2), 111–115.

Raven, P. H., Evert, R. F., & Curtis, H. (1981). *Biology of plants*. New York, NY: Worth Publishers.

Rebecca, O. P. S., Boyce, A. N., & Chandran, S. (2010). Pigment identification and antioxidant properties of red dragon fruit (*Hylocereus polyrhizus*). *African Journal Biotechnology*, *9*(10), 1450–1454.

Sengul, M., Yildiz, H., Gungor, N., Cetin, B., Ezer, Z., & Ercisli, S. (2009). Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. *Pakistan Journal of Pharmaceutical Sciences*, *22*(1), 102–106.

Shewry, P. R., Tatham, A. S., Barro, F., Barcelo P., & Lazzeri, P. (1995). Biotechnology of breadmaking: Unraveling and manipulating the multi-protein gluten complex. *Nature Biotechnology*, *13*(11), 1185–1190.

Tenore, G. C., Novellino, E., & Basile, A. (2012). Nutraceutical potential and antioxidant benefits of red pitaya (*Hylocereus polyrhizus*) extracts. *Journal of Functional Food*, *4*(1), 129–136.

Van Loey, A., Ooms, V., Weemaes, C., Van den Broeck, I., Ludikhuyze, L., Denys, S., & Hendrickx, M. (1998). Thermal and pressure-temperature degradation of chlorophyll in broccoli (*Brassica oleracea* L. *italica*) juice: A kinetic study. *Journal of Agricultural and Food Chemistry*, *46*(12), 5289–5294.

Yamazaki, E., Murakami, K., & Kurita, O. (2005). Easy preparation of dietary fiber with the high water-holding capacity from food sources. *Plant Foods for Human Nutrition*, *60*(1), 17–23.

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Assessment of IRAP Markers to Evaluate the Genetic Diversity of *Eurycoma longifolia*

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ABSTRACT

Eurycoma longifolia, among the more precious medicinal herbs, has many promising benefits. However, the limited information on genetic diversity of the species has hindered many further related studies such as breeding programme. This study aims to evaluate the transferability of retrotransposon-based markers, Inter-Retrotransposon Amplified Polymorphism (IRAP) to evaluate the genetic diversity within and between two selected provenances. Thirteen IRAP markers were initially screened for their transferability properties. Findings showed that retrotransposon elements were present in the genome of *E. longifolia*. Four single primers and two combinations of primers were effectively utilized to analyse the genetic diversity of *E. longifolia*. Genetic diversity assessment indicated that the total diversity (H_T) was 0.2396 and the diversity within the population (H_S) was 0.2233. The coefficient of gene differentiation (G_{ST}) was 0.0680, signifying that there were 6.8% total genetic variations between the provenances and 93.2% variations among individual accessions within the provenances. Thus, the genetic variations between selected provenances were lower than the genetic variations within the provenances.

Keywords: Genetic variations, molecular marker, retrotransposon, transferability

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INTRODUCTION

Eurycoma longifolia or locally known as "Tongkat Ali" is the most prominent herb species in Malaysia, currently receiving great attention from the government and

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people due to its medicinal importance. *E. longifolia* has also been listed as the top five herbs of focus in the National Key Economic Areas (NKEA) in Malaysia (Performance Management and Delivery Unit [PEMANDU], 2013). Many studies have been conducted on the chemical profiling and pharmacological importance such as aphrodisiac (Ang et al., 2000; Low et al., 2013; Mohd Azmi et al., 2004; Subramaniam, 2013), antimalarial (Kuo et al., 2004), and anticancer (Kuo et al., 2004; Wong et al., 2012). Therefore, the market demand for *E. longifolia* in the Malaysian herbs industries is very high.

Nowadays, most of the raw materials of *E. longifolia* needed for the industries especially the root parts are obtained from the natural forest reserve areas. This practice, in the long run, could endanger the plant species diversity and be economically impractical. Thus, in order to cater the problem of providing raw materials of *E. longifolia*, the Forest Research Institute Malaysia (FRIM) (n. d.) has taken an initiative to conduct a research on "Provenance Trial of *E. longifolia*" via a breeding strategy research to provide important breeding information and conservation strategies. In forestry, the term provenance refers to the original place where the plant samples were collected.

The provenance trial is conceptually defined as the selection on a population level with a special type of plantation to understand the well-adapted organisms to various environmental conditions through genetic adaptation and phenotypic plasticity. Provenance variation can be found in any plant species that occupies a broad range of geographic conditions. The variations or differences in environmental conditions result in the adaptation of the plant species to the environment, consequently changing the population's genetic pool. Findings in the provenance trial would provide the best match between specific environmental conditions and the plant species of various origins (Hettasch, 2002).

Provenance trial research has included a wide range of sampling activities of *E. longifolia* throughout Peninsular Malaysia, establishment of the plantation trial to monitor the growth performances and the establishment of provenance resource stands for conservation strategies (Nor Fadilah et al., 2016), screening for significant phytochemical content (Mohd Zaki et al., 2015a, 2015b) and plant pathology study (Wan-Muhammad-Azrul et al., 2018). Breeding strategies of any plant species are improved by complimenting them with the knowledge of the genetic variation at the molecular level.

Molecular markers are used to evaluate the genetic variation within and between provenances. They have proven to provide a precise assessment and identification of plant cultivars (Tnah et al., 2011) compared to biochemical and phenotypic markers which are negatively affected by low polymorphisms (Kalendar et al., 1999). The DNA profiling and genetic information of plant cultivars are very important for the plant improvement programs such as germplasm and conservation management. The best molecular markers have abundant genomic sequence information, high polymorphisms, co-dominant markers to differentiate between homozygotes and heterozygotes, and easily reproducible technique (Tnah et al., 2011).

Therefore, due to the limited information on the genetic diversity of *E. longifolia* in the study of provenance trial, this study aims to evaluate the transferability of retrotransposon-based markers, Inter-Retrotransposon Amplified Polymorphism (IRAP) to analyze the genetic diversity within and between two selected provenances. IRAP relies on PCR amplifications between two retrotransposon insertion sites (Kalendar et al., 1999). The high copy number of retrotransposon elements makes them abundant and prevalent in plant genomes, and gives a strong basis as a marker system. Furthermore, its structure and replication strategy are advantageous as markers. Additionally, this simpler technique uses fewer numbers of markers but has high polymorphism detection, high reproducibility, and high reliability (Guo et al., 2006; Yuying et al., 2011).

MATERIALS AND METHODS

Plant Materials

A total of 56 accessions of *E. longifolia* were collected as test materials; 30 accessions were from Forest Reserve of Kapas Island (FRPK) located in the state of Terengganu, and 26 accessions were from Forest Reserve of Maokil (FRMJ) located in the state of Johor. The provenances for this study were selected based on the promising growth performances in the provenance trial study (data not shown). The GPS coordinates of every mother plants found during the sampling activities were recorded (data not shown). The characteristics of the selected provenances are shown in Table 1.

Table 1
Origin of the provenances (based on sampling locations) characterized by latitude, longitude, altitude and forest type

Provenances	Latitude (N°)	Longitude (E°)	Altitude (m)	Forest type
Forest Reserve of Maokil, Johor	2°01’ - 2°12’	102°49’ - 103°21’	94 – 228	Hill forest
Forest Reserve of Kapas Island, Terengganu	4°35’ - 5°12’	103°15’ - 103°16’	30 - 169	Hill forest (Island)

DNA Extraction, Purification and Quantitation

Genomic DNA (gDNA) was extracted from leaves of each accession using a modified CTAB method (Murray & Thompson, 1980). The gDNA stock was stored at -80˚C. The purification of total gDNA was conducted following the Roche Diagnostic (2008) standard procedure: High Pure PCR Template Preparation Kit. Then, the gDNA and purified gDNA were quantified by NanoDrop 2000 system, UV-VIS Spectrophotometer (Thermo Fisher Scientific). The gDNA and purified DNA were measured at 260 nm, with optical density at 600 nm. The purity of the genomic DNA and purified DNA was calculated at ratios 260/280 nm and 260/230 nm.

Additionally, the quality of extracted gDNA and purified DNA was evaluated by running a gel electrophoresis on 0.8% w/v agarose at 100 volt and 300 mA for 20 minutes. The DNA calf thymus (at the concentration of 5, 10, 15, 25, and 50 ng/µl) was used as a standard marker for the evaluation. The preparation and dilution of the template DNA were conducted based on both results of quantification by NanoDrop 2000 system and bands intensity observed on the agarose gel.

Analysis of IRAP Markers

PCR amplifications were performed in 25 µl total volume consisted of approximately 100 ng genomic DNA, 5X Green GoTaq® Flexi PCR Buffer (Promega Corporation), 200 µM dNTP (Promega Corporation), 3.0 mM $MgCl_2$ (Promega Corporation), 0.8 µM of each primer and 0.05 U of Taq DNA polymerase (Promega Corporation). The PCR (Applied Biosystems® 2720 Thermal Cycler) cycling conditions were as followed: 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 42°C for 1 min (T_a depended on primers used) and 72°C for 1 min; and the final extension step at 72°C for 10 minutes. PCR products were separated in 1.0% agarose gel in 1X TAE buffer for 90 minutes at 60V and 400mA (Agisimanto et al., 2008). The characteristics of retrotransposon-based primers are shown in Table 2.

Data Analysis

For each IRAP fragment, the presence or absence was scored on gel images and binary matrices were assembled in Microsoft Excel spreadsheets. Descriptive statistics including the Number of Scored Band (NSB), Number of Polymorphic Band (NPB), Percentage of Polymorphic Band (PPB), Polymorphism Information Content (PIC), Effective Multiplex Ratio (EMR), Marker Index (MI), and Heterozygosity (*He*) were calculated to determine the informativeness of the marker system.

Assessment of the genetic diversity parameters such as percentage (%) of polymorphism, Nei's genetic diversity (*He*), Shannon's information index (*I*), the observed number of alleles (*Na*), and the effective number of alleles (*Ne*) was done using POPGENE software version 1.32 (Yeh et al., 1999). Determination of genetic structure was also carried out using POPGENE software based on the Nei's method. Total gene diversity (H_T), genetic diversity within the population (H_{TS}), the coefficient of gene differentiation (G_{ST}) and gene flow (N_m) were analyzed.

NTSYS-pc version 2.2 (Rohlf, 2009) was used to perform the cluster analysis. SIMQUAL option was chosen to calculate the genetic similarities based on Jaccard's coefficient. Unweighted Pair Group Method with Arithmetic Average (UPGMA) was employed to construct the dendogram using SAHN module. Graphical 3D image of Principal Component Analysis (PCA) representing genetic diversity was generated using the EIGEN and MOD-3D programs of this software.

Table 2
Characteristics of IRAP primers used for amplifications

No	Primers' name	Retrotransposon name and orientation	Sequence (5'-3')	Tm (°C)	Reference/Source
1.	Copia-F	Ty1 Copia - Forward	5' ACNGCNTTYYTNCAYGG 3'	41.9	Flavell et al. (as cited in Agisimanto et al., 2008, p. 247)
2.	Copia-R	Ty1 copia - Reverse	5' ARCATRTCRTCNACRTA 3'	35.9	Flavell et al. (as cited in Agisimanto et al., 2008, p. 247)
3.	Sabrina-C0945	Sabrina - Forward	5' GCAAGCTTCCGTTTCCGC 3'	47.5	Leigh et al., 2003
4.	Sukkula-9900	Sukkula - Forward	5' GATAGGGTCGCATCTTGGGCGTGAC 3'	57.5	Leigh et al., 2003
5.	Sukkula-91673	Sukkula - Forward	5' TGTGACAGCCCGATGCCGACGTTCC 3'	59.1	Leigh et al., 2003
6.	Sukkula-E0228	Sukkula - Reverse	5' GGAACGTCGGCATCGGGCTG 3'	54.9	Leigh et al., 2003
7.	Nikita-57	Nikita - Forward	5' CGCATTTGTTCAAGCCTAAACC 3'	47.9	Leigh et al., 2003
8.	Nikita-E2647	Nikita - Forward	5' ACCCCTCTAGGCGACATCC 3'	50.3	Leigh et al., 2003
9.	LTR 6150	BARE-1 - Reverse	5' CTGGTTCGGCCCATGTCTATATGTATCCACACATGTA 3'	60.5	Kalendar et al., 1999
10.	LTR 6149	BARE-1 - Forward	5' CTCGCTCGCCCACTACATCAACCGCGTTTATT 3'	60.6	Kalendar et al., 1999
11.	3 LTR	BARE-1 - Forward	5' TGTTTCCCATGCGACGTTCCCCAACA 3'	56.0	Teo et al., 2005
12.	5' LTR 1	BARE-1 - Reverse	5' TTGCCTCTAGGGCATATTTCCAACA 3'	50.9	Teo et al., 2005
13.	5' LTR 2	BARE-1 - Reverse	5' ATCATTCCCTCTAGGGCATAAATTC 3'	48.9	Teo et al., 2005

Note: Tm = melting temperature

RESULTS AND DISCUSSION

Informativeness of the IRAP Markers

Initially, 13 IRAP primers were screened for their transferability for the genetic diversity study of *E. longifolia*. However, only four primers; Copia-F (Figure 1), Nikita-57 (Figure 2), Sabrina-C0945 (Figure 3) and Sukkula-9900 (Figure 4) out of the 13 tested primers yielded polymorphic banding patterns with high resolutions and reproducible bands. Additionally, the two combinations of primers [(Sukkula-9900 + Copia-F) and (Sukkula-9900 + Sabrina-C0945)] successfully yielded unique polymorphic banding patterns out of six combinations primers tested.

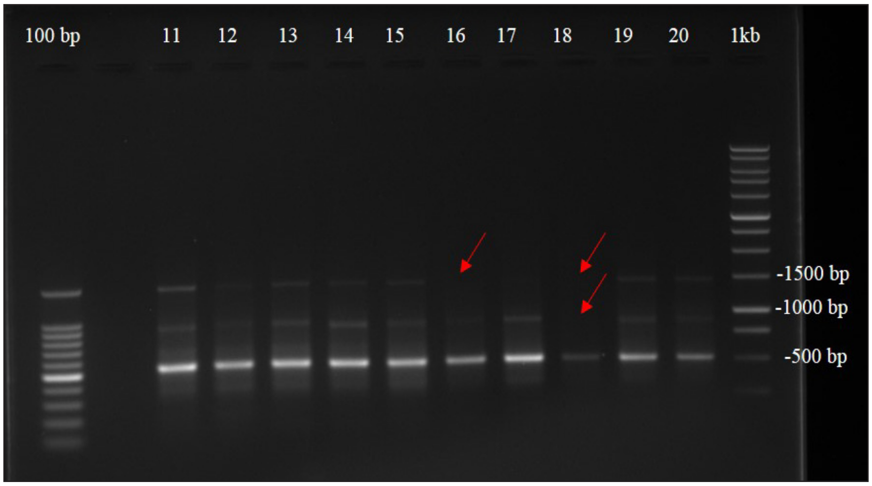

Figure 1. IRAP amplification profile of primer Copia-(F). The accessions shown were provenance of FRPK, accession number 11 to 20. DNA ladder of 100 bp and 1 kb (Promega Corporation) were located on the left and right well. The arrows show variations observed in the bands

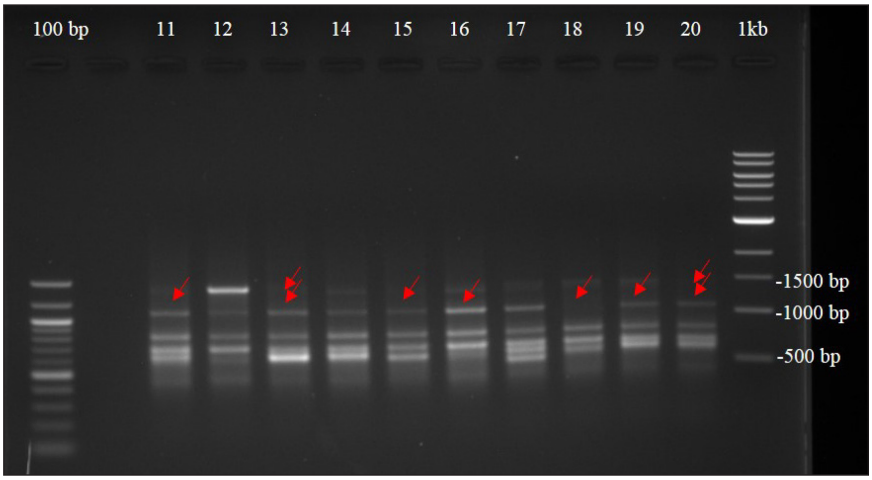

Figure 2. IRAP amplification profile of primer Nikita-57. The accessions shown were provenance of FRMJ, accession number 11 to 20. DNA ladder of 100 bp and 1 kb (Promega Corporation) were located on the left and right well. The arrows show variations observed in the bands

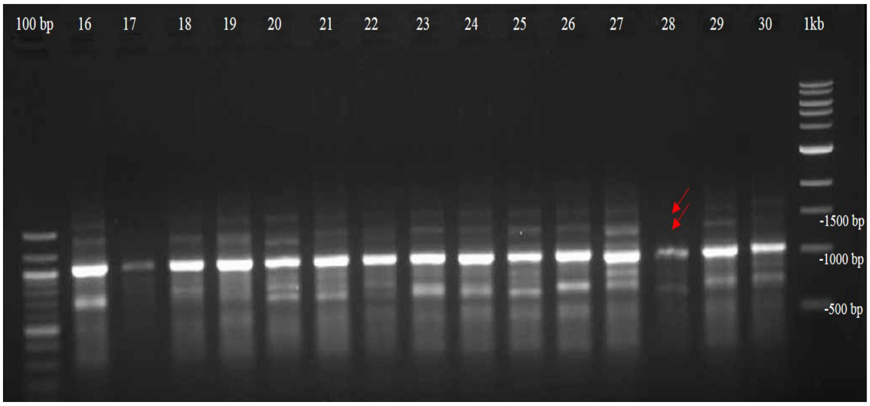

Figure 3. IRAP amplification profile of primer Sabrina-C0945. The accessions shown were provenance of FRMJ, accession number 16 to 30. DNA ladder of 100 bp and 1 kb (Promega Corporation) were located on the left and right well. The arrows show variations observed in the bands

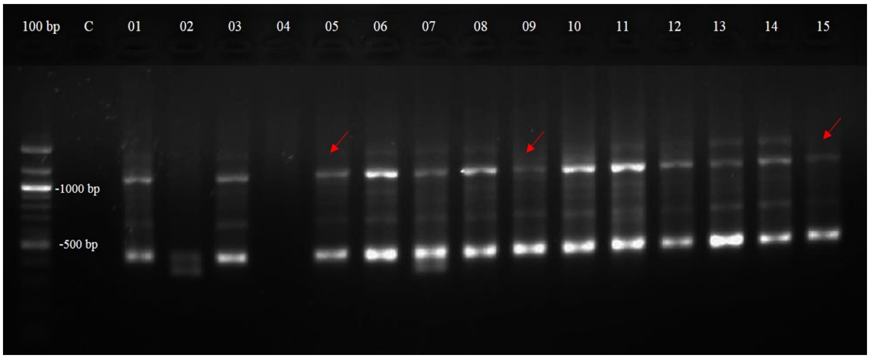

Figure 4. IRAP amplification profile of primer Sukkula-9900. The accessions shown were provenance of FRMJ, accession number 1 to 15 and the C lane shows Control. DNA ladder of 100 bp (Promega Corporation) is located on the left well. The arrows show variations observed in the bands

As a result, a total of 1247 polymorphic bands were scored from the range of 300 – 1500 base pairs (Table 3). The Percentage of Polymorphic Bands (PPB) ranged from 50.0% to 100.0% and the Polymorphism Information Content (PIC) values of the markers ranged from 0.469 to 0.833 with the average of 0.6816 (Table 4). The highest recorded PIC value was by primer Nikita-57 while the lowest PIC value was by the combination of primers Sukkula-9900 and Copia-F. The PIC values for all primers were generally more than 0.50 except for the combination of primers Sukkula-9900 and Copia-F. The Heterozygosity (*He*) values were positively related to PIC values, ranging from 0.565 to 0.852 (Table 4). Then, the Marker Index (MI) was calculated by multiplying PIC value and Effective Multiplex Ratio (EMR) value. The EMR values were positively correlated with the PPB values; thus, the higher the PPB values, the higher the EMR values. The MI values for all the primers tested in this study ranged from 0.68 to 3.72 while the EMR values ranged from 1.00 to 5.00 (Table 4). These criteria, the PPB, PIC, MI, EMR and *He* are usually used to assess the informativeness of the marker system or to evaluate the discriminatory power of a marker.

The retrotransposon families of Copia, Nikita, Sukkula and Sabrina were initially identified in the plant genome of barley, mostly present as solo long terminal repeats (LTRs) (Leigh et al., 2003). Leigh et al. (2003) also successfully identified the efficiency of 15 retrotransposon-based primers in barley for their applications in single-primer IRAP, two-primer IRAP, and REMAP technique. Nikita-57 primer was observed to produce very clear profiles for all the marker techniques (single-primer

Table 3
Comparison of the observed band size (bp) to the expected band size (bp) and optimized annealing temperature [Ta (°C)]

No.	Primers' name	Expected band size range (bp) (Leigh et al., 2003)	Observed band size range (bp)	Ta (°C)
1.	Copia-F	~270	500 - 1500	37.0
2.	Sabrina-C0945	1000 - 4000	800 - 1500	42.5
3.	Sukkula-9900	500 - 4000	300 - 1500	50.5
4.	Nikita-57	1000 - 4000	400 - 1500	42.9
5.	Combination: Copia-F + Sukkula-9900	Non recorded	400 - 1500	43.8
6.	Combination: Sabrina-C0945 + Sukkula-9900	Non recorded	300 - 700	46.5

Table 4
Informativeness assessment of the IRAP markers

No.	Primers' name	NSB	NPB	NMB	PPB	PIC	EMR	MI	*He*
1.	Copia-F	3	2	1	66.7%	0.549	1.33	0.73	0.627
2.	Sabrina-C0945	7	5	2	71.4%	0.819	3.57	2.92	0.839
3.	Sukkula-9900	5	5	0	100%	0.743	5.00	3.72	0.779
4.	Nikita-57	7	5	2	71.4%	0.833	3.57	2.97	0.852
5.	Sukkula-9900 + Copia-F	5	3	2	60.0%	0.469	1.80	0.844	0.565
6.	Sukkula-9900 + Sabrina-C0945	4	2	2	50.0%	0.677	1.00	0.677	0.728
	Total	31	22	9	-	-	-	-	-
	Minimum	3	2	0	50.0%	0.469	1.00	0.73	0.565
	Maximum	7	5	2	100%	0.833	3.57	3.72	0.852
	Mean	5.2	3.7	1.5	69.9%	0.682	2.18	1.98	0.732

Note: NSB – Number of Scored Band, NPB – Number of Polymorphic Band, NMB – Number of Monomorphic Band, PPB – Percentage of Polymorphic Band, PIC – Polymorphism Information Content, EMR – Effective Multiplex Ratio, MI - Markers Index, *He* - Heterozygosity

IRAP, two-primer IRAP and REMAP). In the primer comparison study, the clarity profile was differentiated as poor < clear bands < very clear. The same indication of the clarity profile was also observed from Sukkula-9900 primer. However, the rate of polymorphism of Sukkula-9900 primer ranged from 40 to 80% for all the marker techniques; it was higher compared to Nikita-57 primer which only ranged from 10 to 60%. For Sabrina-C0945 primer, the capacity of clarity profile was indicated as clear, and the rate of polymorphisms observed from all the marker techniques ranged from 10 to 30%.

Findings in this study demonstrated that the retrotransposon elements were present in the genomic DNA of *E. longifolia*. Many studies have also demonstrated that IRAP markers are universal and successful for many tested species to date (Kalendar & Schulman, 2007). Among the advantages of IRAP markers is only a little or no sequence information of the species is needed, meaning that IRAP markers are applicable to be used for any plant species without the prior knowledge of gene sequences. In this study, primer sequences were obtained from the study of barley retrotransposon families (Leigh et al., 2003) because most of the primers used in this study had been utilized for other species in several different studies without the information of the species DNA sequences. Additionally, according to Kalendar et al. (2011) retrotransposon elements found in barley families are abundant, which is more than 50%, in comparison to other plant species (Ragupathy et al., 2013).

For an example, IRAP markers had been utilized for the following plant species; *Triticum* species (Farouji et al., 2015), saffron (Alsayied et al., 2015), Indian potato (Sharma & Nandieneni, 2014), plum (Senkova et al., 2013), persimmon (Yuan et al., 2012), Japanese apricot (Yuying et al., 2011), some exotic plant species from Perm region (Boronnikova & Kalendar, 2010), *Citrus suhuiensis* (Agisimanto et al., 2008), Japanese persimmon (Guo et al., 2006) and even fungus species (Santana et al., 2012). Furthermore, according to Ragupathy et al. (2013), based on the list of retroelements found in the plant genomes that have whole genome sequences (consisting of 32 different species), retrotransposon elements can be found as low as 7.02% in *Arabidopsis thaliana* (weed) and as high as 75.6% in *Zea mays* (maize). However, according to the list, none of the retroelements could be found in *Phoenix dactylifera* (date-palm). Retrotransposon elements were also abundant in eukaryotic genome, therefore making the elements a great choice as molecular markers (Kalendar et al., 2017).

On the other hand, among the advantages of IRAP is the replication strategy also known as "copy and paste" mechanism, in which the transposition occurs via RNA intermediate resulting in new insertions that increases polymorphisms in the genome (Kalendar et al., 1999). The structure of retrotransposon elements is highly conserved. Additionally, the dispersion of the elements is abundant and prevalent, and the markers are also highly reproducible. Grzebelus (2006) and Guo et al. (2006) recorded that both IRAP markers were applicable to evaluate the intraspecific relationship since their markers were extremely polymorphic. Along with the explained characteristics, Alsayied et al. (2015) mentioned that retrotransposon-based markers could be used as indicators in biodiversity assessment.

Genetic Diversity of *Eurycoma longifolia* Provenances

The genetic diversity parameters such as the observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's gene diversity (*He*), Shannon's information index (*Na*), and percentage of polymorphic loci (%) were generated using POPGENE software version 1.32 (Yeh et al., 1999) (Table 5).

The analysis indicated that the observed number of alleles (*Na*) of FRPK (1.65 ±0.486) was higher than and FRMJ (1.55 ±0.506) while the effective number of alleles (*Ne*) of FRPK was 1.43 ±0.422 and FRMJ was 1.37 ±0.398. Nei's gene diversity (*He*) recorded from the provenance of FRPK was 0.238 ±0.221 and FRMJ was 0.209 ±0.215. The Shannon's information index (*I*) reflected the same order as *He* did: FRPK (0.348 ±0.308) and FRMJ (0.306 ±0.307). For the percentage of polymorphic loci (%), both provenances scored more than 50% with FRPK having 64.5% and FRMJ having 54.8% (Table 5).

In the SNPs study, the values of observed heterozygosity (H_0) was lower than the expected heterozygosity (*He*) (Osman et al., 2003). The *He* values generated by IRAP markers in this study were considered comparable to SNPs which ranged from 0.177 to 0.246. However, the limitation for dominant markers such as IRAP is that the data obtained could not calculate the H_0 values. Nonetheless, a study conducted on the genetic diversity of *E. longifolia* in five populations within the province of Riau using RAPD as markers revealed a very similar range of *He* value which ranged from 0.13 to 0.27 (Zulfahmi, 2013).

In a comparison of the *He* values of *E. longifolia* sampled in this study with that of *Shorea leprosula* Miq., which was listed in the Malaysia Plant Red List (Chua et al., 2010), the *He* values recorded in *E.*

Table 5
Genetic diversity of E. longifolia *provenances inferred by IRAP markers*

No.	Prove-nances	Observed number of alleles (*Na*) ±StDev	Effective number of alleles (*Ne*) ±StDev	Nei's gene diversity (*He*) ±StDev	Shannon's information index (*I*) ±StDev	Percentage of polymorphic loci (%)	The number of polymorphic loci
1.	FRPK	1.65 ±0.486	1.43 ±0.422	0.238 ±0.221	0.348 ±0.308	64.5	20
2.	FRMJ	1.55 ±0.506	1.37 ±0.398	0.209 ±0.215	0.306 ± 0.307	54.8	17

longifolia in this study was lower. The study by Lee et al. (2000), utilizing the allozyme markers to assess the genetic diversity of *S. leprosula* recorded that the *He* value for *S. leprosula* sampled throughout Peninsular Malaysia was surprisingly high (0.369 ± 0.025). Nevertheless, it should be noted that allozyme markers are codominant markers whereas IRAP markers in this study are dominant markers. Weising et al. (2005) mentioned that when associating estimation value of *He*, dominant markers data could generally produce the maximum value of 0.5 since only two alleles could be differentiated at each locus while codominant markers could produce up to 1.0 value of *He*. On the other hand, Lee et al. (2000) also mentioned that the value recorded for *S. leprosula* was among the highest *He* recorded for tropical tree species.

Nevertheless, the percentage of polymorphic loci (%) observed in SNP markers ranged from 49.0% to 75.0% as assessed from 47 individuals originated from six populations; this value was also considered comparable to the percentage of polymorphic loci (%) observed in IRAP markers which were 54.8% and 64.5% (Table 5) as assessed from 56 accessions from two provenances. The findings were also similar to the percentage of polymorphic loci (%) observed in Riau province, ranging from 40.9% to 70.5%.

The genetic structure of the two provenances of *E. longifolia* inferred by IRAP markers is revealed in Table 6. The total genetic diversity (H_T) was found to be 0.239 and the diversity within the population (H_S) was 0.223 (Table 6). The value for H_T and H_S as demonstrated by IRAP markers were also parallel to the study in SNPs (0.288 and 0.219, respectively) (Osman et al., 2003) and RAPD in Riau province (0.29 and 0.2, respectively) (Zulfahmi, 2013). Furthermore, the coefficient of gene differentiation (G_{ST}) in this study was found to be 0.068 (Table 6) which was considered as a moderate value following the classification by Nei (1978). According to Nei (1978), the G_{ST} value can be determined by three levels: low when the G_{ST} value is less than 0.05, moderate when the G_{ST} value is in the range of 0.05 to 0.15 and high when the G_{ST} value is more than 0.15. In this study, G_{ST} value signified that there were 6.8% of the total genetic variations between the provenances tested and there were 93.2% variations among individual accessions within the provenances. Both studies using SNPs and RAPD markers showed the high values of G_{ST}, 0.24 (Osman et al., 2003) and 0.31 (Zulfahmi, 2013) respectively, meaning that the genetic variations between the selected populations in the study were lower than genetic variations within populations.

Table 6
Genetic structure of two provenances (FRMJ and FRPK) of E. longifolia *identified by IRAP markers*

H_T = Total diversity	0.239
H_{TS} = Diversity within population	0.223
G_{ST} = Coefficient of gene differentiation	0.068
N_m = Gene flow based on Gst	6.85
Sample size	56

Contrary to the findings by SNPs (Osman et al., 2003) and RAPD (Zulfahmi, 2013), the G_{ST} value by IRAP in this study was lower, indicating higher chances of gene flow probably because of a higher distance of seeds dispersal. However, Osman et al. (2003) mentioned that higher G_{ST} might had resulted from smaller number of individuals sampled per population in the study. Similarly to the conditions of RAPD (Zulfahmi, 2013), the study of genetic diversity in Riau Provinces (Indonesia) also sampled about five individuals per provenances whereas the study by SNPs (Osman et al., 2003) in Malaysia sampled about six to nine individuals per populations. For this study by IRAP markers in two provenances, the sample sizes were 26 and 30 individuals per provenances.

The gene flow (N_m) based on N_m was found to be 6.85 (Table 6). The N_m value observed in this study was considered as high. In comparison to the N_m value of *E. longifolia* in Riau province (N_m = 1.11), the findings by IRAP in two provenances was higher. Govindaraju (1989) classified gene flow values into three levels: high when the N_m value is more than 1, moderate when the N_m value is in the range of 0.25 to 0.99, and low when the N_m value is less than 0.25.

According to the Oxford Dictionary of Biology, conceptually, gene flow is defined as the exchange of genetic materials by interbreeding between populations of the same species or between individuals within a population. Gene flow increases the variation in the genetic composition of a population but decreasing the genetic variations among the populations. Thus, higher gene flow value indicates higher genetic variations ("Gene flow", 2007). In the case of *E. longifolia*, its seeds dispersal is believed to be aided by wild birds. The pulpy layer of the fruits is the main attractive characteristic that appeals the wild birds (FRIM, n. d.). Therefore, since wild birds fly farther, the tendency of seeds dispersal distance increases.

Cluster Analysis of *E. longifolia* Provenances

A UPGMA dendogram is generated based on the Jaccard similarity coefficient and for the dendogram (Figure 5) varied from 0.7 to 0.96. This means that the similarity of the two provenances is about 70 to 96%. Cluster analysis revealed three broad groups where group 1 could be further divided into 3 subgroups, group 2 could be further divided into 5 subgroups, while group 3 only consisted of 2 accessions from FRMJ. However, the results indicated that *E. longifolia* accessions from two provenances were not differentiated by origin. From the dendogram (Figure 5), it could be observed that group 1 and 2 consisted of almost proportionate number of accessions from the provenances of FRPK and FRMJ.

Findings in this study were contradicted with findings by RAPD and SNPs markers on the study of genetic diversity of *E. longifolia* in Malaysia and Indonesia, respectively. In this study, cluster analysis showed that there was no clear indication that the clustering was based on the provenances or the geographic origins. Mohd Razi et al. (2013) found that *E. longifolia* cultivars in Malaysia could be clustered into three clusters in which i) East Malaysia (Sabah), ii) middle part of west Malaysia (Terengganu and Pahang) and iii) west part of East Malaysia (Kedah and Kelantan). Furthermore, Osman et al. (2003) demonstrated that the *E. longifolia* sampled from Johor, Langkawi, Terengganu, Pahang, Melaka and tissue culture collection could be grouped into two major groups with geographical origin as the clustering basis. Additionally, Zulfahmi (2013) on the study of five provinces of *E. longifolia* in Indonesia also further subdivided the five provinces into two major groups.

On the other hand, the study of the genetic diversity of *Shorea leprosula* using allozyme marker by Lee et al. (2000) showed that the cluster analysis of eight populations in Malaysia did not reflect the geographical origin of the populations sampled in the study. According to Lee et al. (2000), this might indicate that the populations of *S. leprosula* sampled were part of a continuous population and fragmentation that might have occurred in the past.

Even though the accessions from the two provenances have high similarities, the dendogram (Figure 5) showed clear differentiation between the two provenances. The distribution by Principle Component Analysis (PCA) also revealed the differentiation of two broad groups with moderate genetic similarities (Figure 6).

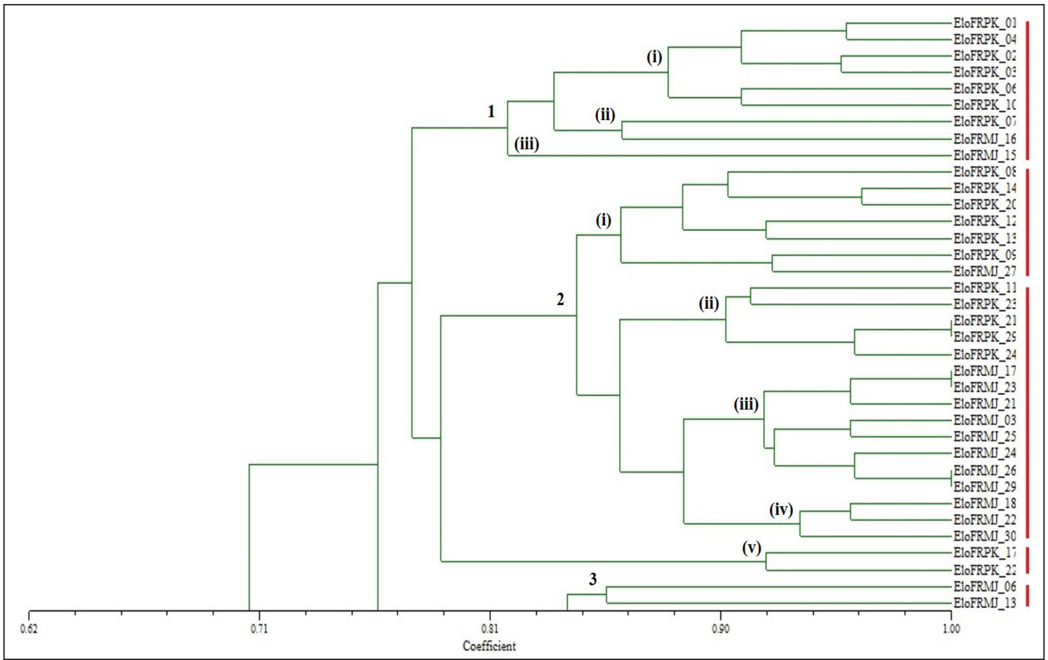

Figure 5. Dendogram constructed from IRAP markers of 56 accessions (FRPK and FRMJ) based on genetic similarity (Jaccard coefficient)

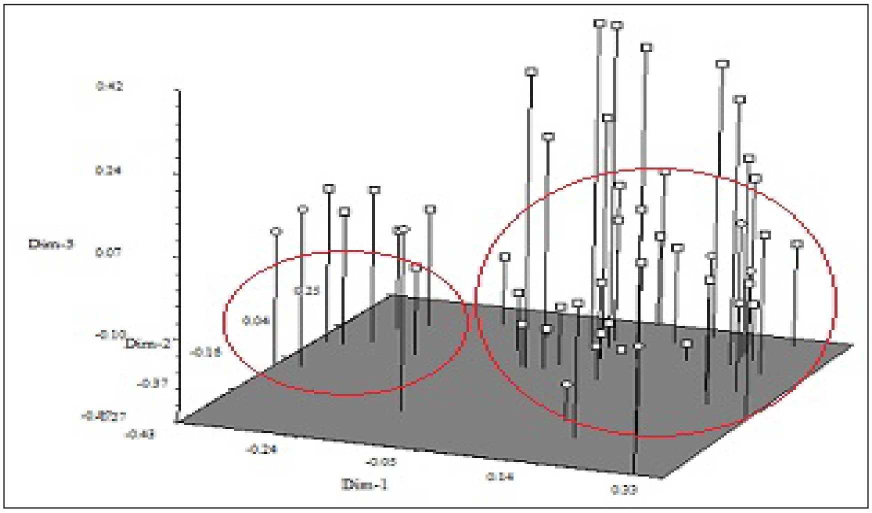

Figure 6. 3D distribution of 56 *E. longifolia* accessions (FRPK and FRMJ) revealed by IRAP markers

CONCLUSION

This study showed that IRAP markers can be effectively used to analyse the genetic diversity of *E. longifolia* based on the informative assessment of the marker system (PPB, PIC, MI, EMR and *He*). The markers are also proven to have the transferability properties, making it interchangeably useable across different species without prior knowledge of gene sequences. The total genetic diversity of two selected *E. longifolia* provenances' were found to be low (0.239). Findings in this study also suggested that there is high gene flow between the provenances (contrary to the previous findings). This implied that the plant breeders may take into consideration the available individuals within the provenances in their breeding strategies and perhaps can overlook the variations between the provenances.

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REFERENCES

Agisimanto, D., Normah, M. N., Ibrahim, R., & Mohamad, A. (2008). *Retros markers for genetic variations in Limau Madu (*Citrus suhuiensis *hort. ex Tanaka)*. Retrieved October 27, 2014, from https://www.pgm-my.org/book/pgm-congress08-proceedings/

Alsayied, N., Fernandez, J. A., Schwarzacher, T., & Heslop-Harrison, J. S. (2015). Diversity and relationship of *Crocus sativus* and its relatives analysed by inter-retroelement amplified polymorphisms (IRAP). *Annals of Botany*, *116*(3), 359–68. DOI: 10.1093/aob/mcv103

Ang, H. H., Cheang, H. S., & Md. Yusof, A. P. (2000). Effects of *Eurycoma longifolia* (Tongkat Ali) on the initiation of sexual performance of inexperienced castrated male rats. *Experimental Animals*, *49*(1), 35–38.

Boronnikova, S. V., & Kalendar, R. N. (2010). Using IRAP markers for analysis of genetic variability in populations of resource and rare species of plants. *Russian Journal of Genetics*, *46*(1), 36–42. doi: 10.1134/S1022795410010060

Chua, L. S. L., Suhaida, M., Hamidah, M., & Saw, L. G. (2010). *Malaysia plant red list (Peninsular Malaysian Dipterocarpaceae)*. Kepong, Malaysia: Forest Research Institute Malaysia.

Farouji, A. E., Khodayari, H., Saeidi, H., & Rahiminejad, M. R. (2015). Genetic diversity of diploid *Triticum* species in Iran assessed using inter-retroelement amplified polymorphisms (IRAP) markers. *Biologia*, *70*(1), 52–60. doi: 10.1515/biolog-2015-0002

Forest Research Institute Malaysia. (n.d.). *Colours of FRIM: The rare sight of fruiting Tongkat Ali*. Retrieved April 30, 2014, from https://www.frim.gov.my/en/the-rare-sight-of-fruiting-tongkat-ali/

"Gene flow". (2007). In *Oxford dictionary of biology* (6^{th} Ed.). Oxford, United Kingdom: Oxford University Press.

Govindaraju, R. D. (1989). Variation in gene flow levels among predominantly self-pollinated plants. *Journal of Evolution Biology*, *2*(3), 173–181. doi: 10.1046/j.1420-9101.1989.2030173.x

Grzebelus, D. (2006). Transposon insertion polymorphism as a new source of molecular markers. *Journal of Fruit and Ornamental Plant Research*, *14*, 21–29.

Guo, D., Zhang, H., & Luo, Z. (2006). Genetic relationships of *Diospyros kaki* Thunb. and related species revealed by IRAP and REMAP analysis. *Plant Science*, *170*(3), 528–533.

Hettasch, M. H., Lunt, K. A., Pierce, B. T., Snedden, C. L., Steyn, D. J., Venter, H. M., & Verryn, S. D. (2002). *Tree breeding course manual*. Pretoria, South Africa: CSIR Environmentek.

Kalendar, R. N, Grob, T., Regina, M., Suoniemi, A., & Schulman, A. H. (1999). IRAP and REMAP: Two new retrotransposon-based DNA fingerprinting techniques. *Theoretical and Applied Genetics*, *98*(5), 704–711.

Kalendar, R. N., Aizharkyn, K. S., Khapilina, O. N., Amenov, A. A., & Tagimanova, D. S. (2017). Plant diversity and transcriptional variability assessed by retrotransposon-based molecular markers. *Journal of Genetics and Breeding*, *21*(1), 128–134.

Kalendar, R. N., Flavell, A. J., Ellis, T. H. N., Sjakste, T., Moisy, C., & Schulman, A. H. (2011). Analysis of plant diversity with retrotransposon-based molecular markers. *Heredity*, *106*(4), 520–530.

Kalendar, R., & Schulman, A. H. (2007). IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. *Nature Protocols*, *1*(5), 2478–2484. doi: 10.1038/nprot.2006.377

Kuo, P. C., Damu, A. G., Lee, K. H., & Wu, T. S. (2004). Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia. Bioorganic and Medicinal Chemistry*, *12*(3), 537–544.

Lee, S. L., Wickneswari R., Mahani, M. C., & Zakri A. H. (2000). Genetic diversity of a tropical tree species, *Shorea leprosula* Miq. (Dipterocarpaceae), in Malaysia: Implications for conservation of genetic resources and tree improvement. *Biotropica*, *32*(2), 213–224. doi: 10.1111/j.1744-7429.2000.tb00464.x

Leigh, F., Kalendar, R. N, Lea, V., Lee, D., Donini, P., & Schulman, A. H. (2003). Comparison of the utility of barley retrotransposon families for genetic analysis by molecular marker techniques. *Molecular Genetics and Genomics*, *269*(4), 464–474.

Low, B. S., Choi, S. B., Abdul Wahab, H., Das, P. K., & Chan, K. L. (2013). Eurycomanone, the major quassinoid in *Eurycoma longifolia* root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis. *Journal of Ethnopharmacology*, *149*(1), 201–207.

Mohd Azmi, M. I., Ahmad Fauzi, P., & Norini, H. (2004). Economic analysis of *Eurycoma longifolia* (Tongkat Ali) harvesting in Peninsular Malaysia. In C. Y. Shyun, M. Mohtar, & N. M. Yunos (Eds.), *Tongkat Ali, Kacip Fatimah and Pegaga new dimensions in complementary healthcare: Proceedings of the seminar on medicinal plants, 20-21 August 2002* (pp. 3-6). Kepong, Malaysia: Forest Research Institute Malaysia.

Mohd Razi, A. R., Abdul Aziz, A., Syed Alwi, S. S. B., & Aziz Ramlan, A. (2013). Relationships between Malaysians cultivars of Tongkat Ali (*Eurycoma longifolia* Jack.) obtained through RAPD analysis. *International Journal of Biotechnology for Wellness Industries*, *2*(1), 45-50.

Mohd Zaki, A., Nor Fadilah, W., Abdul Rashid, L., Nurnadiah, R., Mohd Radzi, A., Mohamad Lokmal, N., … Mohamad, O. (2015a). Preliminary phytochemical screening of polysaccharides content for selection of high quality planting materials: *Eurycoma longifolia*. *Jurnal Teknologi*, *77*(24), 101–105.

Mohd Zaki, A., Nor Fadilah, W., Mohd Radzi, A., Sui Kiong, L., Ab. Rasip, A. G., Mohamad Lokmal, N., … Regina Mariah, J. (2015b). Preliminary phytochemical screening of eurycomanone for selection of high quality planting materials: *Eurycoma longifolia*. *Malaysian Applied Biology*, *44*(1), 23–25.

Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, *8*(19), 4321–4325.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, *89*(3), 583–590.

Nor Fadilah, W., Mohd Zaki, A., & Mohamad Lokmal, N. (2016). Breeding strategies of *Eurycoma longifolia*: Present and future. In B. J. Chee, M. Mastura, K. Getha, M. G. H. Khoo, J. Mailina, M. A. Adiana, & M. S. Roshan Jahn (Eds.), *Proceedings of the 14th Seminar on Medicinal and Aromatic Plants* (pp. 18–23). Kepong, Malaysia: Forest Research Institute Malaysia.

Osman, A., Jordan, B., Lessard, P. A., Norwati, M., Mohd Rosli, H., Norifiza, M. R., … Housman, D. E. (2003). Genetic diversity of *Eurycoma longifolia* inferred from single nucleotide polymorphisms. *Plant Physiology*, *131*(1), 1294–1301.

Performance Management and Delivery Unit. (2013). *Economic Transformation Programme (ETP) annual report 2012*. Retrieved August 25, 2013, from http://etp.pemandu.gov.my/annualreport/upload/Eng_ETP2012_Full.pdf

Ragupathy, R., You, F-M., & Cloutier, S. (2013). Arguments for standardizing transposable element annotation in plant genomes. *Trends in Plant Science*, *18*(7), 367–376.

Roche Diagnostic. (2008). *High pure PCR template preparation kit: Rapidly purify genomic DNA for diverse applications*. Retrieved August 25, 2013, from https://lifescience.roche.com/documents/High-Pure-PCR-Template-Preparation-Kit.pdf

Rohlf, F. J. (2009). *NTSYSpc: Numerical taxonomy system (Version. 2.21c)*. New York, NY: Exeter Software.

Santana, M. F., De Araujo, E. F., De Souza, J. T., Mizubuti, E. S. G., & De Queiroz, M. V. (2012). Development of molecular markers based on retrotransposons for the analysis of genetic variability in *Moniliophthora perniciosa*.

European Journal of Plant Pathology, *134*(3), 497–507. doi: 10.1007/s10658-012-0031-4

Senkova, S., Ziavroska, J., Bezo, M., Stefunova, V., & Razna, K. (2013). Utilization of IRAP technique for plums genotypes differentiation. *Bioscience Research*, *10*(1), 1–7.

Sharma, V., & Nandineni, M. R. (2014). Assessment of genetic diversity among Indian potato (*Solanum tuberosum* L.) collection using microsatellite and retrotransposon-based marker systems. *Molecular Phylogenetics and Evolution*, *73*, 10–17. doi: 10.1016/j.ympev.2014.01.003

Subramaniam, V. (Ed.) (2013). *Malaysian herbal heritage*. Kepong, Malaysia: Forest Research Institute Malaysia.

Teo, C. H., Tan, S. H., Ho, C. L., & Faridah, Q. Z. (2005). Genome constitution and classification using retrotransposon-based markers in the orphan crop banana. *Journal of Plant Biology*, *48*(1), 96–105.

Tnah, L. H., Lee, C. T., Lee, S. L., Ng, K. S., Ng, C. H., & Hwang, S. S. (2011). Microsatellite markers of an important medicinal plant, *Eurycoma longifolia* (Simaroubaceae), for DNA profiling. *American Journal of Botany*, *98*(5), e130–e132. doi: 10.3732/ajb.1000469

Wan-Muhammad-Azrul, W. A., Mohd-Farid, A., Lee. S. Y., Sajap, A. S., Omar, D., & Mohamed, R. (2018). A survey on the occurrence of pests and diseases in Tongkat Ali (*Eurycoma longifolia*) plantations in Peninsular Malaysia. *Journal of Tropical Forest Science*, *30*(3), 362-375. doi: 10.26525/jtfs2018.30.3.362375

Weising, K., Nybom, H., Wolff, K., & Kahl, G. (2005). *DNA fingerprinting in plants: Principles, methods, and applications* (2nd ed.). Boca Raton, USA: Taylor and Francis Group.

Wong, P. F., Cheong, W. F., Shu, M. H., Teh, C. H., Chan, K. L., & Abu Bakar, S. (2012). Eurycomanone suppresses expression of lung cancer cell tumor markers, prohibitin, annexin 1 and endoplasmic reticulum protein 28. *Phytomedicine*, *19*(2), 138–144.

Yeh, F. C., Yang, R-C., & Boyle, T. (1999). *POPGENE version 1.31: Microsoft Window-based freeware for population genetic analysis (Quick user guide)*. Retrieved June 24, 2016, from https://sites.ualberta.ca/~fyeh/popgene.pdf

Yuan, L., Zhang, Q., Guo, D., & Luo, Z. (2012). Genetic differences among 'Luotian-tianshi' (*Diospyros kaki* Thunb.) genotypes native to China revealed by ISSR and IRAP markers. *Scientia Hortuculturae*, *137*, 75–80.

Yuying, S., Xiajun, D., Fei, W., Binhua, C., Zhihong, G., & Zhen, Z. (2011). Analysis of genetic diversity in Japanese apricot (*Prunus mume* Sieb. et Zucc.) based on REMAP and IRAP molecular markers. *Scientia Horticulturae*, *132*, 50–58.

Zulfahmi, R. (2013). Genetic diversity of *Eurycoma longifolia* Jack. based on random amplified polymorphic DNA marker. *Journal of Tropical Forest Management*, *19*(2), 138–144.

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Genome Wide Association Studies for Fatty acids, Mineral and Proximate Compositions in Groundnut (*Arachis hypogaea* L.) Seeds

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ABSTRACT

Groundnut is basically grown for its oil in most countries and the quality of the oil depends on the total oil, protein and fatty acid compositions in the seeds. The objective of this study was to identify markers that were associated with fatty acids, minerals and proximate composition in groundnut seeds. One hundred and seventy groundnut collections were evaluated in the dry season of 2017 at the research field of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Bayero University Kano. Marker trait association was calculated using the GAPIT package via the KDCompute interface. Significant differences were observed between the genotypes for all the trait measured except for moisture content, crude fiber, crude fat, crude protein, dry matter and nonadenic acid. The heritability values of traits ranged from 0.04 to 0.48. A total of 144 highly significant ($P<0.001$) MTAs with 46 markers for fatty acids (118), minerals (4) and proximate (22) compositions were identified. Most of the markers identified possible MTA in both the A and B genomes. Validation studies are needed to find if these markers are identifying one locus or perhaps a locus duplicated in the two genomes.

Keywords: Groundnut, fatty acids, marker trait association, minerals, proximate content

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an essential oil, food and feed crop (Janila et al., 2013b) and it is cultivated in over 27.94 million ha with a total production of 47.1 million tons in 2017 (Food and Agriculture Organization of the United Nations Statistical Databases [FAOSTAT], 2017). The crop is ranked fifth in terms of oil among crops in the world (FAOSTAT, 2014). It is a rich source of protein, fat, minerals, and vitamins. In most countries, groundnut is principally cultivated for its oil but the demand for groundnut as wholesome food has been increasing due to the health benefits associated with consumption of the nutrient-dense peanut kernels (Upadhyaya et al., 2012b). Groundnut is normally used for oil extraction for eatable and industrial purposes which quality depends on the total oil, protein and fatty acid compositions in the seeds. Oleic acid, a monounsaturated fatty acid, and linoleic acid, a polyunsaturated acid, together with a saturated palmitic acid constitute the major bulk (>90%) of fatty acids in groundnut (Upadhyaya et al., 2012b). Oleic acid enhances the shelf life of groundnut products and have other health benefits (Carlson, 1995; Frankel, 1991; Fraser et al., 1997; Upadhyaya et al., 2012b). Groundnut cultivars with high oleic and oil content for oil extraction and those with high oleic and high protein content for groundnut products are preferred, but the efforts to breed for such cultivars are lacking especially in developing countries due to insufficient genetic variability for the traits (Upadhyaya et al., 2012b).

Micronutrient malnutrition as a result of Fe and Zn deficiencies alone affect over 3 billion people around the world as indicated by Upadhyaya et al. (2012a). The widespread of micronutrient malnutrition has led to huge negative socioeconomic impact that cut across all levels of society (Darnton-Hill et al., 2005; Stein, 2010). Efforts at ICRISAT and other places have led to the identification or development of groundnut cultivars with variation in protein, oil content and quality (Upadhyaya et al., 2012b). However, there are no intensive efforts to identify sources of essential minerals such as Fe and Zn (Upadhyaya et al., 2012a). There is therefore a need to develop nutrition rich groundnut cultivars that will meet the demands of mostly developing countries. Identification of markers that are linked to nutritional traits in groundnut will help in fast tracking breeding process for release of nutritional enhanced groundnut cultivars. The identified markers can be used for marker assisted selection. With the development of genomic tools, marker assisted breeding has been used to improve efficiency of selection for traits of interest in groundnut (Agarwal et al., 2018; Janila et al., 2013a; Pandey et al., 2012, 2014;Varshney et al., 2013). The objective was to identify markers that are associated with fatty acids, minerals and proximate composition in groundnut seeds.

MATERIALS AND METHODS

The study was carried out on the research field of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) at

Bayero University Kano, Nigeria. One hundred and seventy groundnut collection were evaluated for fatty acids, minerals and proximate compositions in the 2017 dry season. The experimental design used was randomized incomplete alpha-lattice (10 x 17) with three replications. Each plot consisted of single row measuring 5 m with inter and intra row spacing of 75 cm and 10 cm, respectively. There was 1 m alley between replications. A total of 40 seeds were planted on each row. One seed was planted per hole at a spacing of 10 cm between holes. Basal application of NPK was done to all plots at the rate of 20 kg ha^{-1} N, 40 kg ha^{-1} P_2O_5 and 40 kg ha^{-1} K_2O. Hand weeding was done at 3, 8, and 12 weeks after planting (WAP) to prevent weed infestation and competition. The field was irrigated to provide optimum growth.

Biochemical Analysis

A total of fifty (50) lines were selected from the 170 genotypes based on cluster analysis. From the clusters, 20 high, 10 medium and 10 low yielding varieties in addition to 10 check varieties were selected for the biochemical analysis. The proximate and mineral compositions of the lines were determined using the Buck Scientific Atomic Absorption Spectrophotometer following the Standard Official Method of Analysis procedures described by Association of Official Analytical Chemists (AOAC) (1984, 1994, 1996). Briefly, 0.5g of each sample was weighed into a 50 ml beaker and 10 ml of an acid mixture of nitric acid and per chloric acid in the ratio 2:1 was added to the content in the beaker and placed on a hot plate to undergo digestion at 105˚C for about 20 minutes until the colour changed to colourless. The digest was allowed to cool and made up to 25 ml with distilled water. The 25 ml was introduced into the Buck Scientific Atomic Absorption Spectrophotometer model 210/211 VGP to determine the concentration of the element in the digest. Fatty acid determination was carried out using gas chromatography. Briefly, 3g of each sample was weighed and soaked in 10ml of N-hexane for two days after which the samples were filtered and weighed. Oil extracted from the filtration was collected into a vacuum tube and covered. The oil was weighed into glass vial and capped; 4.0 ml of petroleum ether and 0.5 ml of Na-methoxide were also added and shaken to dissolve. This was allowed to stand for 1-2 hours till a clear solution was formed. Acid formed in the process was neutralized by sodium glyceroxide and the solution was then pipetted out and injected into the gas chromatographic system (7890B GC) for measurement of fatty acids.

Analysis of variance was performed using PROC GLM in Statistical Analysis System (SAS 9.3) using RANDOM statement with the TEST option.

DNA Extraction and DArT Sequencing

Groundnut leaves of 50 genotypes were collected into 96 deep well samples collection plates and sent to Integrated Genotyping Service and Support (IGSS) platform located at Biosciences Eastern and Central Africa (BecA-ILRI) Hub in

Nairobi for genotyping. DNA extraction was done using NucleoMag® plant genomic DNA extraction kit. The genomic DNA extracted was in the range of 50-100ng/µl. DNA quality and quantity were checked on 0.8% agarose. Libraries were constructed according to Kilian et al. (2012). DArTSeq complexity reduction method through digestion of genomic DNA and ligation of barcoded adapters was done followed by PCR amplification of adapter-ligated fragments. Libraries were sequenced using single read sequencing runs for 77 bases. Next generation sequencing was carried out using Hiseq2500.

DArTseq markers scoring was achieved using DArTsoft version 14, which is an in-house marker scoring pipeline based on algorithms. Two types of DArTseq markers were scored, SilicoDArT markers (scored as presence or absence, 1, 0) and biallelic SNP markers which were both scored for presence of the reference allele, the alternative allele, or both in genomic representation of the sample. Both SilicoDArT markers and SNP markers were aligned to the reference genomes of *Arachis duranensis* (V14167, A-genome ancestor) and *A. ipaensis* (K30076, B-genome ancestor) to identify chromosome.

Linkage Disequilibrium and Marker Trait Association

Linkage disequilibrium between SNPs on each chromosome was measured using TASSEL 5.0 (Bradbury et al., 2007). Marker trait association analysis, probability values and % of the effect of these markers were calculated using the GAPIT package via the KDCompute interface (https://kdcompute.igss-africa.org/kdcompute/home). SNPs with MAF <5% and missing data >20% were excluded. Missing values were imputed using the choice of nearest neighbour algorithm using TASSEL 5.0 (Bradbury et al., 2007). We used the unweighted pair-group method to cluster the lines and form a dendrogram using KDCompute.

RESULTS

Biochemical Analysis of the Groundnut Genotypes

The heritability values of traits ranged from 0.04 for crude fat and palmitic acid to 0.48 for linoleic acid. Oleic acid had a heritability of 0.47. Significant differences were observed between the genotypes for all the trait measured except for moisture content, crude fiber, crude fat, crude protein, dry matter and nonadenic acid (Table 1).

Table 1
Means, minimum, maximum and heritability values for chemical compositions of the groundnut genotypes

Traits	Mean	Minimum	Maximum	Heritability
		Fatty acids		
Stearic acid (%)	0.5**	0	3.3	0.34
Lauric acid (%)	0.1**	0	0.9	0.2
Palmitic acid (%)	0.0**	0	0.1	0.04
Oleic acid (%)	2.7**	0	19.1	0.47

Table 1 *(continue)*

Traits	Mean	Minimum	Maximum	Heritability
Linoleic acid (%)	8.3**	0.1	40.8	0.48
Nonadenic acid (%)	0.1ns	0	0.2	0.08
Arachidic acid (%)	0.0**	0	0.2	0.05
Behenic acid (%)	0.1**	0	0.4	0.12
Tricosanoic acid (%)	0.0**	0	0.1	0.04
Tridecanoic acid (%)	0.1**	0	0.9	0.22
		Minerals		
Iron (mg/kg)	145.0**	77	298	0.39
Zinc (mg/kg)	46.0**	30	77.3	0.37
		Proximate		
Moisture (%)	7.3ns	5.7	9	0.43
Crude fibre (%)	4.3ns	4	4.7	0.44
Crude fat (%)	43.4ns	12.2	48.1	0.04
Ash (%)	2.2**	1	4	0.13
Crude protein (%)	20.6ns	16.1	28	0.16
Carbohydrate (%)	22.3**	15.5	45.9	0.19
Dry matter (%)	93.0ns	91	98	1

**=significant at 0.01 level of probability, NS= non-significant at 0.05

Marker Data

The DArTseq genotyping produced 3591 biallelic SNP markers of which 3396 had a call rate that exceeded 0.6999 and the call rate ranged from 0.37 to 1 (Table 2). Of the 3396 markers, just 396 had a minor allele frequency that exceeded 0.05. The average polymorphism information content of the 3396 markers ranged from 0.006 – 0.499. A principal component analyses of the data from the 3124 markers assigned to a chromosome (s) did not reveal a strong discernible population structure in the first PC that accounted for 61% of the variation.

Table 2
Summary of biallelic SNP marker data

Biallelic SNP	Minimum	Maximum
Allele count A	1	118
E-value A	9.65E-29	2.62E-10
Allele count B	1	100
E-value B	1.18E-28	4.35E-10
Polymorphic information content (PIC)	0.006	0.499
Call rate	0.374	1
One ratio SNP	0.006	1
Frequency of homozygous	0.006	0.994
Frequency of heterozygous	0.006	0.799

The DArTseq genotyping produced 12,693 dominant silico markers all with a call rate that exceeded 0.80 (Table 3). Just 2349 (18.5%) of these had a MAF > 0.05. The average polymorphism information content of the 2349 markers ranged from 0.01 – 0.5. Over 76% of the markers aligned with both the A and B genomes were assigned to homeologous chromosomes and the correlation of their position on those two set of homologues was 0.91. Cluster analysis of the markers revealed three discernible groups (Figure 1).

Table 3
Summary of biallelic SNP marker data

Silico markers	Minimum	Maximum
Allele count A	1	49
E-value A	9.65E-29	2.62E-10
Allele count B	1	52
E-value B	1.18E-28	4.35E-10
Polymorphic information content (PIC)	0.011	0.5
Call rate	0.804	1
One ratio SNP	0.006	0.994
Average Read Depth	5	820
Reproducibility	0.95	1

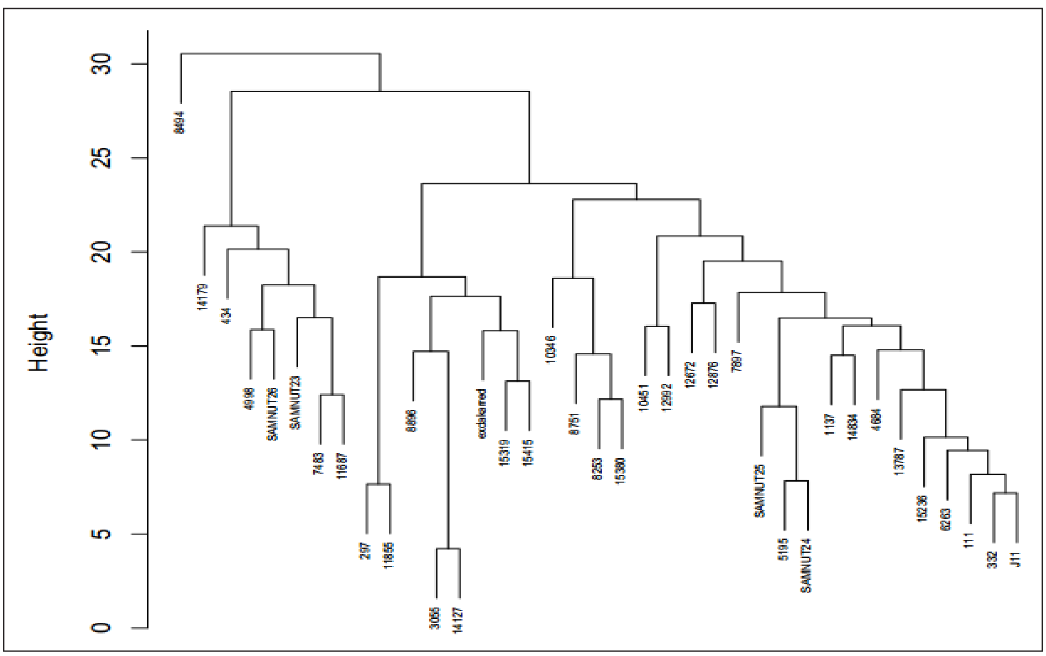

Figure 1. Dendrogram from unweighted pair-group clustering of groundnut accessions using marker data

Linkage Disequilibrium

Linkage disequilibrium (LD) analysis revealed the presence of 305,919 loci pairs. About 36.26% of loci pairs were in significant LD (*P* < 0.05). Further, 9,592 (3.14%) of the pairs were in complete LD (r^2 = 1). There was rapid decline in LD with distance and the correlation analysis revealed negative correlation (*r* = -0.149) between the LD (R^2) and the physical distance; as well as between the P-value and R^2 (*r* = -0.751), revealing the existence of linkage decay (Figure 2).

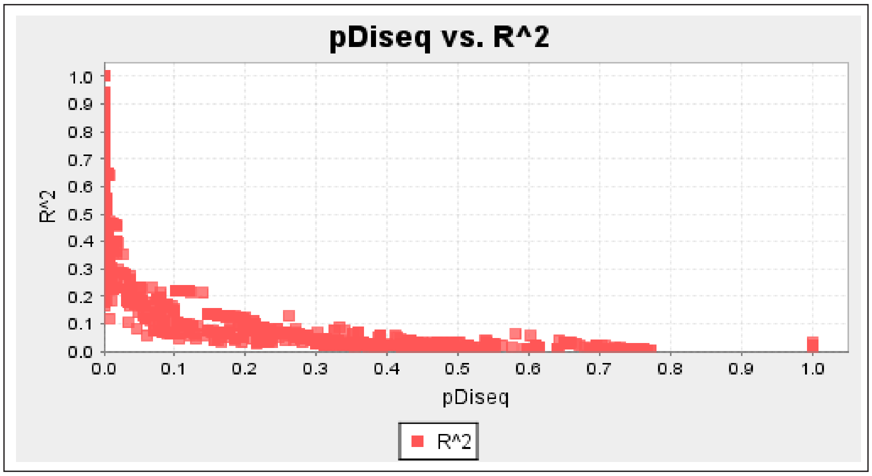

Figure 2. Scatter plot showing association between linkage disequilibrium (r^2) and probability of r^2 (pDiseq)

Marker Trait Association

A total of 144 highly significant (P<0.001) MTAs with 46 markers for fatty acids (118), minerals (4) and proximate (22) compositions were identified (Table 4 and Supplementary Table 1, Supplementary Figure 1 and Supplementary Table 2). Oleic acid (OA) had 16 MTAs with 8 markers that are common to both A and B genomes (Supplementary Figure 1) and explained about 53 – 59 % of the phenotypic variance (PV) observed. Linoleic acid (LNA) had 26 MTAs with 13 markers common to both A and B genomes. Eight out the 13 markers were similar to the markers associated to OA with similar positions in the chromosomes where they were identified (Supplementary Table 1). The markers explained 42 – 58 % of the observed PV for LNA. Two MTAs with one marker was identified for lauric acid (LA) and nonadenic acid (NA). The markers explained about 55 and 98 % of the PV observed for LA and NA, respectively. Numbers of MTAs identified for stearic acid (SA) were 26 with 13 markers each for A and B genome. Of the 13 markers identified, 12 were common to both A and B genome all the markers explained 42 – 64 % of the observed PV. Arachidic acid (AA) had 22 MTAs with 11 markers each on A and B genome which explained 41 – 58 % of the PV. Twenty four (24) MTAs were identified for tricosanoic acid (TA) and each genome had 12 MTAs with 12 common markers that explained 60 – 66 % of PV. All the MTAs identified were equally distributed on the A and B genomes. Markers, M1 – M13 had significant associations with LNA, SA and AA, while OA had significant associations with M1 – M8 (Supplementary Table 1). Most of the markers were identified on two or more chromosomes and most chromosomes had two or more markers associated to them. No significant MTAs (P>0.001) were identified for palmitic and behenic acids.

For iron (Fe) and zinc (Zn), four MTAs (p<0.001) were identified for Fe while no MTA (p>0.001) was identified for Zn. The markers explained 54 – 56 % of the PV for Fe and two markers were identified on chromosome A08. Significant MTAs was identified for only dry matter (DM) among all the proximate compositions determined. Twenty two MTAs were identified for DM with 10 markers on the A genome and 12 markers on the B genome. The 10 markers on the A genome were the same markers on the B genome with two additional markers and the markers explained 36 – 39 % of the PV observed.

Table 4
Marker-trait associations (MTAs) identified for fatty acids, minerals and proximate composition of groundnut seeds

SN	Trait	No. of MTAs	P. value range	Rsquare range
		Fatty acids composition		
1	Oleic	16	0.000268-000578	0.529-0.585
2	Linoleic	26	0.000181-0.000835	0.424-0.583
3	Palmitic	-	-	-
4	Lauric	2	0.000263-0.000265	0.545-0.552
5	Nonadenic	2	0.0000176-0.0000178	0.976-0.977
6	Stearic	26	0.0000663-0.000754	0.415-0.643
7	Arachidic	22	0.000195-0.000983	0.409-0.578
8	Tricosanoic	24	0.000257-0.000454	0.597-0.656
9	Behenic	-	-	-
		Minerals		
10	Iron	4	0.000797-0.00093	0.542-0.556
11	Zinc	-	-	-
		Proximate		
12	Ash	-	-	-
13	Carbohydrate	-	-	-
14	Crude fiber	-	-	-
15	Crude Protein	-	-	-
16	Crude fat	-	-	-
17	Dry matter	22	0.000746-0.000989	0.361-0.394
18	Moisture	-	-	-

DISCUSSION

The significant differences obtained among the 50 selected groundnut genotypes indicate that genetic variation exists among the genotypes for the traits studied. The significant variability observed for the main fatty acids such as oleic and linoleic acids as well as other fatty acids suggests that sufficient variability exists for the genetic improvement of essential fatty acids in groundnut. Janila et al. (2014) reported genetic variability for Fe and Zn concentrations in groundnut seeds. No significant difference was observed between the groundnut for moisture content but the mean and maximum value were below the average 14% recommended by Waliyar et al. (2015).

The marker data suggested that the population was not highly structured and more markers were produced in the B genome than the A genome. Many polymorphic markers were detected with large portion having MAF < 0.05 with average PIC values of about 0.07. Bertioli et al. (2016) had indicated that groundnut had a low polymorphism rate and low genetic diversity. The linkage disequilibrium (LD) declined with distance and probability. Pandey et al. (2014) and Mwadzingeni et al. (2017) had earlier reported rapid LD decay with distance.

The marker trait association (MTAs) studies revealed 144 significant MTAs (p <0.001) involving 46 markers. Most of the markers identified possible MTA in both the A and B genomes. Validation studies will be needed to see if these markers are identifying one locus or perhaps a locus duplicated in the two genomes.

Pandey et al. (2014) used SSR markers and identified some MTAs for oil, oleic acid, protein and zinc content. These MTAs were located majorly on chromosome A06 and B06 and explained up to 40% of the PV. In our study, some markers were identified for OA, LNA, AA, TA and DM on chromosome A06 and B06 and the markers explained more than 90% of the observed PV which doubled what was reported by Pandey et al. (2014). This may be possible because SNP markers are more informative than SSR markers. Despite the similarities of the reported chromosomes by Pandey et al. (2014) with ours, there are no supporting evidence that the positions are similar. Zhang et al. (2018) identified four MTAs with three markers for OA and three MTAs with markers for LNA. The MTAs were located on chromosome A09, A10 and B08, and the markers were similar for both OA and LNA and located in the same position. In our study we also identified MTAs on chromosome A09, A10 and B08. The markers associated to both OA and LNA which constitute more than 80% of the fatty acids in groundnut were similar and in the same position as also reported by Zhang et al. (2018). The results suggest some possible associations between these traits and may explain why OA:LNA ratio increases with increase in oleic acid percentage as reported by Upadhyaya et al. (2012b) and Zhang et al. (2018). There are many minor effect QTLs, or genes controlling oleic acid and linoleic acid in groundnut, including the major gene FAD2. In our study, 13 markers were associated with both oleic acid and linoleic acid.

The only available reported MTAs for Fe and Zn was that of Pandey et al. (2014). They identified one MTA for Zn and no MTA for Fe. In our study, we identified four MTAs for Fe and no MTA was identified for Zn at $p<0.001$ but two MTAs with one similar marker on chromosome A04 and B04 at $p=0.0048$ which was above the threshold set for identifying MTA were observed. It is important to also report that Pandey et al. (2014) reported one MTA for Zn on chromosome B04 which is similar to our findings. We could not validate if these markers are identifying one locus or perhaps a locus duplicated in the two genomes because it is only one marker but identified on both chromosomes.

From the result of the MTAs analysis, most of the MTAs identified on the A subgenome were also identified on the respective homeologous chromosome on the B subgenome. Agarwal et al. (2018) had shown that significant proportion of marker loci that were assigned to chromosome of one subgenome were mapped to respective homeologous positions on chromosomes of the other subgenome. Quantitative traits are usually complex and controlled by multiple genes that often have individually small effects (Upadhyaya & Nigam, 1999), and we detected considerable large number of markers for most of the traits because of the large density of markers used in our study. Only a few related markers were detected in the study by Zhang et al. (2018) due to the low density of tested markers.

CONCLUSION

The present study identified a total of 144 highly significant marker trait associations involving 46 markers for nine traits out of the 18 studied traits. Seven fatty acids had significant MTAs while mineral and proximate compositions had one significant MTAs each. The markers identified in this study can serve as useful genomic resources to initiate marker-assisted selection and trait introgression of groundnut for improvement of nutritional and biochemical compositions of groundnut. Further studies are required to validate the significant markers identified in the present study using a larger population.

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REFERENCES

Agarwal, G., Clevenger, J., Pandey, M. K., Wang, H., Shasidhar, Y., Chu, Y., … Varshn, B. (2018). High-density genetic map using whole-genome resequencing for fine mapping and candidate gene discovery for disease resistance in peanut. *Plant Biotechnology Journal*, *16*(11), 1954–1967. doi: 10.1111/pbi.12930

Association of Official Analytical Chemists. (1984). *Official methods of analysis: Association of Official Analytical Chemists* (14th ed.). Gaithersburg, USA: AOAC International.

Association of Official Analytical Chemists. (1994). *Official methods of analysis: Association of Official Analytical Chemists* (16th ed.). Gaithersburg, USA: AOAC International.

Association of Official Analytical Chemists. (1996). *Official methods of analysis: Association of Official Analytical Chemists* (16th ed.). Gaithersburg, USA: AOAC International.

Bertioli, D. J., Cannon, S. B., Froenicke, L., Huang, G., Farmer, A. D., Cannon, E. K., … Ozias-Akins, P. (2016). The genome sequences of *Arachis duranensis* and *Arachis ipaensis*, the diploid ancestors of cultivated peanut. *Nature Genetics*, *48*(4), 438–446. doi: 10.1038/ng.3517

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633–2635. doi: 10.1093/bioinformatics/btm308

Carlson, S. E. (1995). The role of PUFA in infant nutrition. *Inform*, *6*(8), 940–946.

Darnton-Hill, I., Webb, P., Harvey, P. W., Hunt, J. M., Dalmiya, N., Chopra, M., … Ball, M. J. (2005). Micronutrient deficiencies and gender: Social and economic costs. *American Journal of Clinical Nutrition*, *81*(5), 1198S–1205S. doi: 10.1093/ajcn/81.5.1198

Food and Agriculture Organization of the United Nations Statistical Databases. (2014). *Crops processed*. Retrieved March 23, 2019, from http://www.fao.org/faostat/en/#data/QD

Food and Agriculture Organization of the United Nations Statistical Databases. (2017). *Crops*. Retrieved March 23, 2019, from http://www.fao.org/faostat/en/#data/QC

Frankel, E. N. (1991). Recent advances in lipid oxidation. *Journal of the Science of Food and Agriculture*, *54*(4), 495–511.

Fraser, G. E., Sumbureru, D., PribiEw, P., Neil, R. L., & Frankson, M. A. C. (1997). Association among health habits, risk factors, and all-cause mortality in a black California population. *Epidemiology*, *8*(2), 168–174. doi: 10.1097/00001648-199703000-00008

Janila, P., Nigam, S. N., Pandey, M. K., Nagesh, P., & Varshney, R. K. (2013a). Groundnut improvement: Use of genetic and genomic tools. *Frontiers in Plant Science*, *4*(2), 1–16. doi: 10.3389/fpls.2013.00023

Janila, P., Venuprasad, R., Abhishek, R., U., A., Reddy, K. R., Waliyar, F., & Nigam, S. N. (2013b). Genetic analysis of resistance to late leaf spot in interspecific groundnuts. *Euphytica*, *193*(1), 13–25. doi: 10.1007/s10681-013-0881-7

Janila, P., Varshney, R. K., Pandey, M. K., Nigam, S. N., Sudini, H., Gowda, M. V. C., … Nagesh, P. (2014). Iron and zinc concentrations in peanut (*Arachis hypogaea* L.) seeds and their relationship with other nutritional and yield parameters. *The Journal of Agricultural Science*, *127*(5), 1771–1781. doi: 10.1017/S0021859614000525

Kilian, A., Wenzl, P., Huttner, E., Carling, J., Xia, L., Blois, H., … Uszynski, G. (2012). Diversity arrays technology: A generic genome profiling technology on open platforms. In. F. Pompanon & A. Bonin (Eds.), *Data production and analysis* *in population genomics: Methods in molecular biology* (pp. 67-89). Totowa, USA: Humana Press.

Mwadzingeni, L., Shimelis, H., Rees, D. J., & Tsilo, T. J. (2017). Genome-wide association analysis of agronomic traits in wheat under drought-stressed and non-stressed conditions. *PLoS One*, *12*(2), e0171692. doi: 10.1371/journal.pone.0171692

Pandey, M. K., Monyo, E., Ozias-Akins, P., Liang, X., Guimaraes, P., Nigam, S. N., … Varshney, R. K. (2012). Advances in Arachis genomics for peanut improvement. *Biotechnology Advances*, *30*(3), 639–651. doi: 10.1016/j.biotechadv.2011.11.001

Pandey, M. K., Upadhyaya, H. D., Rathore, A., Vadez, V., Sheshshayee, M. S., Sriswathi, M., … Varshney, R. K. (2014). Genomewide association studies for 50 agronomic traits in peanut using the “reference set” comprising 300 genotypes from 48 countries of the semi-arid tropics of the world. *PLOS One*, *9*(8), e105228. doi: 10.1371/journal.pone.0105228

Stein, A. J. (2010). Global impacts of human mineral malnutrition. *Plant and Soil*, *335*(1), 133–154.

Upadhyaya, H. D., Dronavalli, N., Singh, S., & Dwivedi, S. L. (2012a). Variability and stability for kernel iron and zinc contents in the icrisat mini core collection of peanut. *Crop Science*, *52*(6), 2628. doi: 10.2135/cropsci2012.05.0306

Upadhyaya, H. D., Mukri, G., Nadaf, H. L., & Singh, S. (2012b). Variability and stability analysis for nutritional traits in the mini core collection of peanut. *Crop Science*, *52*(1), 168. doi: 10.2135/cropsci2011.05.0248

Upadhyaya, H., & Nigam, S. N. (1999). Detection of epistasis for protein and oil contents and oil quality parameters in peanut. *Crop Science*, *39*(1), 115–118.

Varshney, R. K., Mohan, S. M., Gaur, P. M., Gangarao, N. V, Pandey, M. K., Bohra, A., … Gowda, C. L. (2013). Achievements and prospects of genomics-assisted breeding in three legume crops of the semi-arid tropics. *Biotechnology Advances*, *31*(8), 1120–1134. doi: 10.1016/j.biotechadv.2013.01.001

Waliyar, F., Osiru, M., Ntare, B. R., Kumar, K. V. K., Sudini, H., Traore, A., & Diarra, B. (2015). Post-harvest management of aflatoxin contamination in groundnut. *World Mycotoxin Journal*, *8*(2), 245–252. doi: 10.3920/Wmj2014.1766

Zhang, X., Zhu, S., Zhang, K., Wan, Y., Liu, F., Sun, Q., & Li, Y. (2018). Establishment and evaluation of a peanut association panel and analysis of key nutritional traits. *Journal of Integrative Plant Biology*, *60*(3), 195–215. doi:10.1111/jipb.12601

APPENDIX

Supplementary Table 1

Supplementary information for markers with significant p values

Trait	SNP	Chromosome	Position	P.value	Rsquare
Oleic	M1	A09	16232985	0.000268	0.58456
	M2	A08	37481891	0.000292	0.577639
	M3	A09	4215070	0.000313	0.572082
	M4	A08	5671449	0.000322	0.569869
	M5	A06	10156052	0.000328	0.568253
	M6	A07	4811754	0.000333	0.567016
	M7	A10	11042529	0.00041	0.550656
	M8	A06	87914790	0.000496	0.535798
	M1	B09	21044599	0.000316	0.575865
	M2	B08	23091556	0.000346	0.568617
	M3	B09	5249774	0.000377	0.561866
	M4	B07	110098705	0.000382	0.561002
	M5	B06	45124360	0.000396	0.558062
	M6	B07	4744979	0.000397	0.557931
	M7	B10	17082028	0.000508	0.538937
	M8	B06	109255383	0.000578	0.529013
Linoeic	M1	A09	16232985	0.000181	0.582842
	M2	A08	37481891	0.000182	0.582553
	M3	A09	4215070	0.000187	0.579766
	M4	A08	5671449	0.000197	0.574978
	M5	A06	10156052	0.000206	0.571331
	M6	A07	4811754	0.000207	0.570598
	M8	A06	87914790	0.000211	0.568902
	M7	A10	11042529	0.00024	0.557678
	M9	A10	100626921	0.000656	0.471341
	M10	A02	93813091	0.000693	0.466757
	M11	A10	78514031	0.000724	0.463168
	M12	A06	110005450	0.000778	0.457348
	M13	A03	105924455	0.000835	0.451567
	M1	B09	21044599	0.000191	0.554382
	M2	B08	23091556	0.000192	0.553852
	M3	B09	5249774	0.000198	0.551163
	M4	B07	110098705	0.00021	0.545689
	M5	B06	45124360	0.000218	0.54213
	M6	B07	4744979	0.00022	0.54116
	M8	B06	109255383	0.000221	0.540816
	M7	B10	17082028	0.000255	0.527179
	M9	B10	126223500	0.000619	0.447052
	M10	B02	108681196	0.00065	0.442763
	M11	B10	103607600	0.000765	0.428652
	M13	B03	107485963	0.000776	0.427451
	M12	B06	134748296	0.000804	0.424307
Lauric	M14	A09	110528207	0.000263	0.545145
	M14	B09	146236499	0.000265	0.552669
Nonadenic	M15	A09	109069638	1.77E-05	0.976985
	M15	B09	146924640	1.78E-05	0.976232

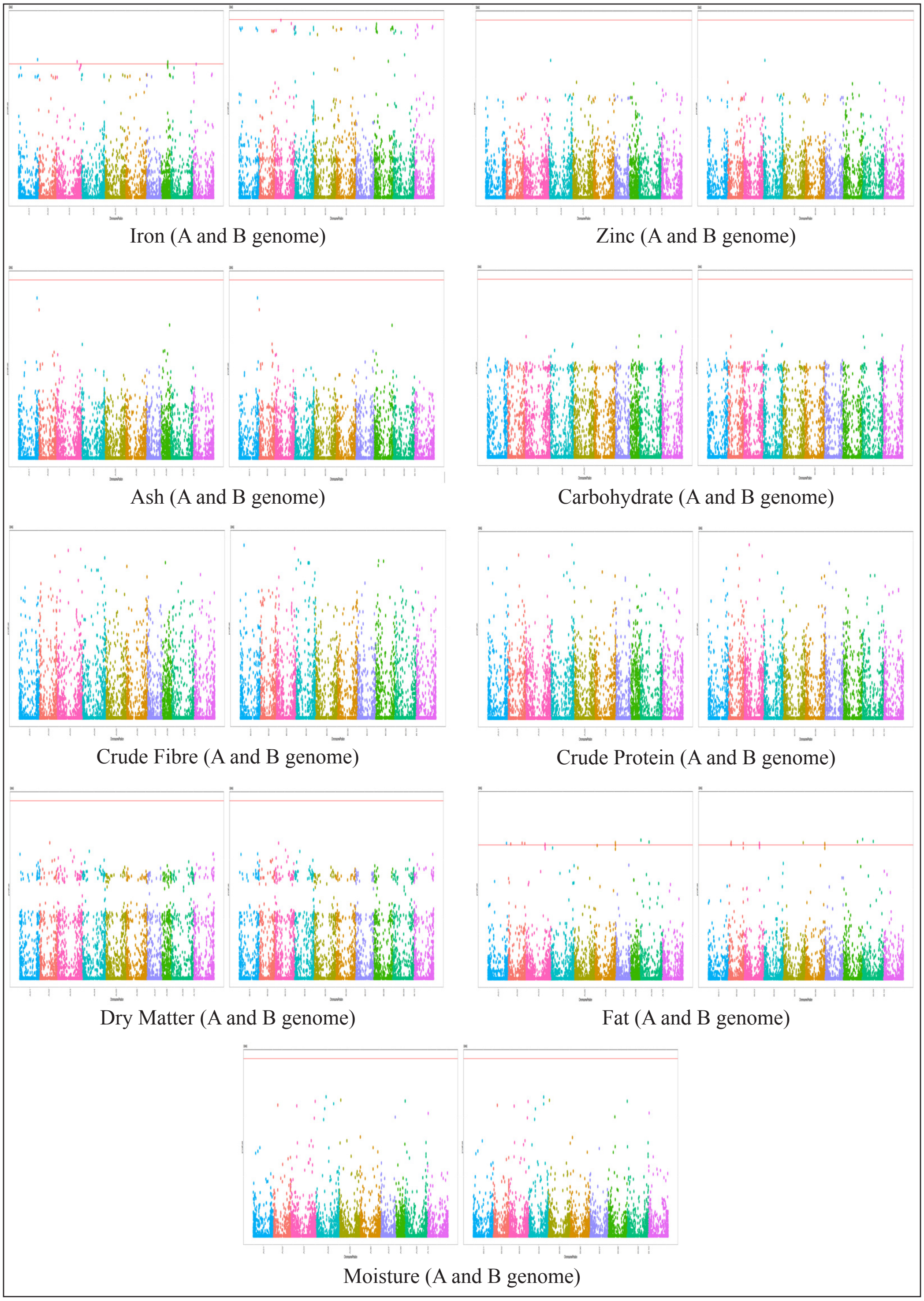
Supplementary Table 1 *(continue)*

Trait	SNP	Chromosome	Position	P.value	Rsquare
Stearic	M2	A08	37481891	6.63E-05	0.642489
	M1	A09	16232985	6.79E-05	0.640277
	M3	A09	4215070	6.95E-05	0.638011
	M4	A08	5671449	7.14E-05	0.635537
	M8	A06	87914790	7.27E-05	0.633824
	M5	A06	10156052	7.50E-05	0.630931
	M6	A07	4811754	8.15E-05	0.62302
	M7	A10	11042529	8.78E-05	0.616146
	M11	A10	78514031	0.000165	0.558755
	M16	A07	40769959	0.000428	0.476145
	M13	A03	105924455	0.000512	0.461203
	M9	A10	100626921	0.000687	0.437158
	M10	A02	93813091	0.000754	0.429647
	M2	B08	23091556	9.54E-05	0.574868
	M1	B09	21044599	9.74E-05	0.572849
	M3	B09	5249774	9.88E-05	0.571403
	M8	B06	109255383	0.000101	0.569034
	M17	B07	110098705	0.000105	0.565681
	M5	B06	45124360	0.000108	0.562159
	M6	B07	4744979	0.000121	0.551313
	M7	B10	17082028	0.000128	0.545474
	M11	B10	103607600	0.000248	0.48204
	M16	B06	52503651	0.000361	0.446955
	M13	B03	107485963	0.000416	0.43384
	M9	B10	126223500	0.000487	0.419706
	M10	B02	108681196	0.000515	0.414651
Arachidic	M1	A09	16232985	0.000195	0.577966
	M8	A06	87914790	0.000196	0.576992
	M2	A08	37481891	0.000204	0.572817
	M17	A08	5671449	0.000234	0.557492
	M3	A09	4215070	0.00024	0.554658
	M7	A10	11042529	0.00025	0.550454
	M5	A06	10156052	0.000251	0.549865
	M6	A07	4811754	0.000263	0.544914
	M11	A10	78514031	0.000582	0.461265
	M18	A07	40769959	0.000726	0.438816
	M13	A03	105924455	0.000983	0.408672
	M1	B09	21044599	0.000238	0.566744
	M8	B06	109255383	0.000254	0.560106
	M2	B08	23091556	0.000257	0.558556
	M3	B09	5249774	0.000299	0.54269
	M4	B07	110098705	0.000303	0.541401
	M7	B10	17082028	0.000312	0.538333
	M5	B06	45124360	0.000315	0.537222
	M6	B07	4744979	0.000332	0.531868
	M18	B06	52503651	0.000735	0.451595
	M11	B10	103607600	0.000768	0.447314
	M13	B03	107485963	0.000937	0.428048
Tricosanoic	M19	A01	92846737	0.000257	0.655511

Supplementary Table 1 *(continue)*

Trait	SNP	Chromosome	Position	P.value	Rsquare
	M20	A07	880042	0.000332	0.628646
	M21	A03	31817347	0.000335	0.627809
	M22	A04	106555766	0.000341	0.625965
	M23	A09	1400546	0.000348	0.623736
	M24	A02	24356338	0.000352	0.622545
	M25	A04	93590112	0.000388	0.612734
	M26	A03	129975580	0.000394	0.61098
	M27	A04	53129067	0.000416	0.605431
	M28	A04	45718128	0.000431	0.602066
	M29	A03	96432676	0.00045	0.597596
	M30	A05	8095276	0.000454	0.596717
	M19	B06	106755490	0.000257	0.655434
	M20	B07	620457	0.000332	0.628566
	M21	B03	35192532	0.000335	0.627716
	M22	B03	8173213	0.000341	0.625905
	M23	B09	1681755	0.000349	0.623606
	M24	B02	28213282	0.000353	0.622426
	M25	B04	102341064	0.000388	0.612621
	M26	B03	130875833	0.000395	0.610817
	M27	B04	102428625	0.000417	0.605288
	M28	B04	45429705	0.000431	0.602022
	M29	B01	135265182	0.000451	0.597406
	M30	B05	8494827	0.000454	0.596589
Iron	M31	A01	101409239	0.000797	0.555483
	M32	A03	111434595	0.000893	0.548444
	M33	A08	34891811	0.000901	0.547903
	M34	A08	34361128	0.000993	0.541932
Dry Matter	M35	A09	5710373	0.000774	0.394268
	M36	A09	49143701	0.000863	0.384995
	M37	A06	110497678	0.000884	0.38301
	M38	A02	75296891	0.000907	0.380853
	M39	A01	99031328	0.000913	0.380295
	M40	A02	89569235	0.000926	0.379134
	M41	A02	14768065	0.000945	0.377409
	M42	A06	110512576	0.00095	0.37699
	M43	A03	103594754	0.000954	0.376603
	M44	A03	104218069	0.000989	0.37362
	M35	B09	2764950	0.000746	0.384769
	M36	B09	75930448	0.000834	0.375085
	M37	B08	97153563	0.000851	0.373381
	M39	B02	19708590	0.000872	0.371217
	M38	B05	136889865	0.00089	0.369448
	M43	B03	105334523	0.000903	0.368263
	M40	B02	103154677	0.000917	0.366917
	M42	B06	135281181	0.000921	0.366569
	M41	B02	17949139	0.000941	0.364662
	M44	B03	105920615	0.000967	0.3624
	M45	B02	19667396	0.000974	0.361721
	M46	B06	135672991	0.000988	0.36052

Supplementary Figure 1. Manhattan plots of marker trait associations of fatty acids



Supplementary Figure 2. Manhattan plots of marker trait associations for minerals and proximate composition

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Response of *Cayratia trifolia* towards Pb, NaCl, Diesel and Wounding Stresses through Expression of a *CtSRG1* Gene

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ABSTRACT

By-products of the petroleum industry contaminate the environment, hence decreasing the soil fertility. However, in some contaminated areas, plants such as *Cayratia trifolia* continue to survive despite the harsh environment. Therefore in this study we aim to investigate the survival of *C. trifolia* at a petroleum refinery, PETRONAS Penapisan Melaka Sdn. Bhd (PPMSB). The main objective of this study was to identify and characterize candidate gene involved in the stress response. Differential display approach was performed on *C. trifolia* grown on soil and sludge, to identify up-regulated and down-regulated partial cDNAs. Out of 23 cDNAs checked, 18 genes were up-regulated genes while five were down-regulated. A partial gene (244 bp) represented by DEG7 fragment has a high similarity to the translocon. Blast2GO analyses was performed and showed DEG7 as a gene responsible for stress responses. A fragment of 1371 bp named as the *CtSRG1* gene was succesfully amplified using the combination of RACE-PCR and degenerate PCR amplification. BlastN and BlastX analyses indicated that the *CtSRG1* gene had higher similarity to translocon. CtSRG1 protein consists of Rieske and SRPBCC ligand-binding domains. The expression profile of *CtSRG1* gene using qRT-PCR showed up-regulated expression when treated with Pb, NaCl and diesel but down-regulated with wounding treatment.

Keywords: Diesel, gene isolation, NaCl, Pb, stress-responsive gene, wounding

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INTRODUCTION

The root structure of a plant growing in normal and ideal environment tends to establish sufficient relationship with the soil matrix thus allowing an adequate supply of oxygen and water. However, if the roots grow in contaminated environments such as in petroleum sludge, the soil particles tend to be covered with a hydrophobic layer. This reduces the availability of water and requires high oxygen concentrations to survive hence creating a condition called water stress (Peña-Castro et al., 2006). Subsequently, the hydrophobic nature of the pollutant leads to various other abiotic stresses (Gogosz et al., 2010). Overall, the presence of environmental stresses in the sludge can interfere with the various systems in the plant, primarily preventing their absorption of water and nutrients (Kirchmann et al., 2017).

Petroleum sludge causes many adverse impacts on living organisms. Numerous studies have shown that plants grown around the area of petroleum sludge cannot thrive long. For example, germination and growth of *Vigna unguiculata* seedling was retarded in the area of high sludge concentrations (Sangeetha & Thangadurai 2014). In addition, Morales et al. (2012) showed that sludge also contained high concentrations of salt that consequently caused reduction of ion absorption including chloride (Cl^-) in rice. Sludges also contain heavy metals which can cause gradual decline in seedling height (Issoufi et al., 2006).

Interestingly, a liana plant known as *Cayratia trifolia* from the Vitaceae family was found to survive in areas contaminated with petroleum sludge (Singh et al., 2012). *Cayratia trifolia* is commonly known as 'bush grape', 'fox-grape', 'lakum' in Malaysia, 'kalit-kalit' in Philippines, 'galing-galing' in Indonesia, 'ta det' in Laos, 'thao kan khaao' in Vietnam and 'vualai' in Papua New Guinea (Gupta et al., 2012). Phytochemical investigation showed that the leaf of *C. trifolia* plants contained high levels of secondary metabolites such as kaempferol, myricetin, quercetin, triterpenes and epifriedelanol (Ragasa et al., 2014). The plant is widely used in traditional medicine for the treatment of wounding, anemia, stomachic diseases and diabetic. Additionally, the plant is reported to possess antibacterial, antiviral, antifungal, antiprotozoal, hypoglycemic, anticancer and diuretic activities (Kumar et al., 2011). Thus, *C. trifolia* was selected for this study because of its ability to defend itself when facing the stressful conditions created by the presence of petroleum sludge.

However, to date, there is still no conclusive molecular study to describe how the plant adapts in the harsh environment area. At a molecular level, stress defence in plants usually is influenced by a group of stress responsive genes (Agarwal et al., 2013). There are many stress responsive genes that function in controlling homeostasis when plants undergo stress involving ions shifts in the cell due to stress solute such as Pb, NaCl and diesel. When the plant is under environmental stresses, a polypeptide translocation across cell membrane occurs to balance the internal environmental condition of the plant cells (Pogson et al., 2015). These genes are also needed to ensure balance and efficiency of protein importation during abiotic stress response (Sjuts et al., 2017). Therefore, this study was performed to investigate the molecular factors involved in the unique ability described.

MATERIALS AND METHOD

Plant Preparation

Cayratia trifolia plants growing in a petroleum refinery, PPMSB, Melaka, Malaysia were collected. Plants that were collected from sampling site were planted in greenhouse owned by Universiti Kebangsaan Malaysia, Selangor, Malaysia. The plants were placed in a white tank with a dimension of 2m x 1m x 0.3m. The plants were left to grow and reproduce for two months until the mother plants produce F1 generation was produced that was used for this study.

After two months of acclimatization process, *C. trifolia* was treated with Pb, NaCl, diesel and were also wounded. For each experiment, three replicates were undertaken for data accuracy. Plumbum (II) nitrate, $Pb(NO_3)_2$ was used throughout the experiments to induce Pb stress. Stock solutions of $Pb(NO_3)_2$ were prepared using distilled water, and diluted to Pb concentrations of 0.01, 0.3 and 0.6 g/kg. Whereas sodium chloride (NaCl) was used for salt treatment. NaCl solution with the concentration of 50, 100 and 300 mM were prepared by dissolving NaCl pellets with distilled water. As for diesel stress, fuel was obtained from a local gas station, PETRONAS. The diesel solution was mixed with acetone (1:1) as a solvent, and the final concentrations of 40, 70 and 100 g/kg were prepared by diluting the diesel solution with distilled water. Pb and NaCl treatments are carried out for 48 hours and the leaves were then harvested after 1, 24 and 48 hours of the treatments. Meanwhile, diesel treatment is carried out for 14 days where the *C. trifolia* were harvested after day 1, 7 and 14 of the treatments. Wounding stress of *C. trifolia* was carried out by crushing the leaves across the center of the vein using a sharp razor blade.

The leaves were then harvested after 1, 3, 6, 24 and 48 hours of the treatments. Experimental time intervals followed suggestion by Afzal et al. (2011), AL-Jobori et al. (2012), Park et al. (2011) and Shavrukov (2012) with some modifications. All these treatments are carried out separately on different plants.

Total RNA Isolation and Differential cDNA Display

Total RNA of *C. trifolia* was extracted using RNeasy Plant Mini Kit (Qiagen, Germany) according to the supplier's recommendation with some modification by (Yazid & Sidik, 2011). RNA extracted was used for first strand cDNA synthesis using SuperScript III FirstStrand Synthesis System for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) (Invitrogen, USA) according to the manufacturer recommendations. Differential cDNA display was performed using GeneFishing™ DEG Premix Kit (Seegene) according to the instruction provided.

Cloning of Differentially-Displayed cDNA Fragments Into Cloning Vector

The differentially expressed (DEG) bands were individually excised out from the gel by using a QIAquick Gel Extraction Kit (QIAGEN, Germany), and were directly cloned into a yT&A cloning vector (Yeastern Biotech, Taiwan) according to manufacturer's protocol. Polymerase chain reaction (PCR) amplification was performed in a volume of 20 µl of 10× PCR Buffer (Promega) containing dNTP mix (10 mM), magnesium chloride (25 mM), *Taq* DNA polymerase (2.5 U), M13 forward primer (5'- GTTTTCCCAGTCACGAC-3') (10 pM) and M13 reverse primer (5'-GTCA TAGCTGTTTCCTGTGTGA) (10 pM). PCR was performed under the following conditions: preliminary denaturation (3 min, 95ºC), then 29 cycles of denaturing step (1 min, 95ºC), annealing step (1 min, 58ºC), extension (45 sec, 72ºC) and a final extension (10 min, 72ºC). As an additional screening step, the recombinant plasmids were double digested with *Eco*RI and *Bam*HI restriction enzymes (FastDigest®, Fermentas).

Functional Annotation and Classification

To provide insights into the putative function of the genes, all DEG sequences were compared with the non-redundant (NR) and UniProt databases (filter: E-value < 1e-5). The NR annotations of the resulting unigenes were searched with Blast2GO Gene Ontology (GO) functional classification algorithms. This software was used for GO functional classification for DEGs and to determine the distribution of the gene functions of species.

Stress-responsive Gene Isolation

The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used in the amplification of 3' ends of *CtSRG1*. The experiments were conducted according to the recommendation protocols of the supplier using a set of primers, universal primer (UPM) '-AAGCAGTGGTATCAACGCAG AGT-3') and 17FGSP (5'-CGCTTCATGGGGGAGTG CGCTCGAGGTCG-3'). UPM primer was provided with the kit while the 17FGSP primer was designed from the original sequence of the DEG7 fragment. Meanwhile, a PCR-based approach was used to amplify the 5' region using the degenerated primers, PDGF1 (5'-GTKGTKTGGGAYAGGAAYGAR-3') and PDGR1 (5'-TTTGCXGCTT CAAXXTGGXTX-3'). The RACE-PCR cycles for amplification were as follows; 5 cycles of 94°C for 30 sec and 72°C for 3 min: followed by 5 cycles of 94°C for 30 ses, 70°C for 30 sec and 72°C for 3 min; then 25 cycles of 94°C for 30 sec and 68°C for 30 sec; followed by a 3 min final extension at 72°C. Meanwhile, the PCR degenerate amplification were; pre-denaturing at 95°C for 3 min, followed by 95°C for 30 sec, 55°C for 1 min and 72°C for 45 sec for 35 cycles, then followed by a final extension at 72°C for 10 min.

DNA Sequencing and Analyses

Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) and sent for sequencing. The sequences were edited with BIOEDIT software. Multiple sequence alignments were conducted by CLUSTALW (http://www.genome.jp/tools/clustalw/). The sequences were analysed with the Blast program and the NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/).

Quantitative Real-Time PCR (qRT-PCR) for Gene Expression

Total RNA was extracted from different treatment of *C. trifolia* leaves (Pb, NaCl, diesel and wounding) as described above. The qRT-PCR was done using iQ5 Real-time PCR System (BioRad, USA) and the amplifications were performed in a 25 µl reaction containing QuantiFast SYBR GreenPCR Master Mix (2X) (Qiagen, Germany), 10 µM of specific primers and 100 ng of cDNA. The amplification conditions were set as follows: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 10 sec and 59°C for 30 sec. Dissociation curve was established at the end of each run to check the specificity of amplification. For each treatment of *C. trifolia*, three independent biological replicates were used for qRT-PCR analysis. Primer efficiencies were calculated using the standard curves generated by five different concentrations of cDNA prepared from four-fold serial dilution. The gene expression data was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The data obtained represented the fold change (increase or decrease) of the target gene in the treated sample relative to the control sample. The expression level of *CtSRG1* was then normalized against two housekeeping genes, including β-tubulin and elongation factor (EF1α).

RESULTS

Differential cDNA display approach was the first step used to identify differentially expressed genes in the samples of *C. trifolia* grown on two different conditions, on soil and on sludge. A total of 23 cDNA fragments were differentially expressed, where 18 of them were up-regulated whereas the other five were down-regulated (Table 1). The up-regulated gene fragments expressed in cDNA of *C. trifolia* from the sludge treatment were labeled as U, and the down-regulated gene fragments from the soil treatment were labeled as D. The specific DEG selection to the stress responsive genes is an important factor in the next analysis. During initial selection, BLASTX analysis was performed for all DEG sequences by comparing the highest homology matches with some other protein sequences in the NCBI database. All DEGs representing proteins from the BLASTX analysis were classified in the putative function of the gene based on the results of the literature. All analyzed DEG sequences have specific functions in the plant system (Table 2). Among all these, DEG7 indicates the function involved in the response to the stresses. DEG7 protein shows a high similarity to the translocon protein involved in the stress response.

All 14 DEG proteins that underwent classification BLASTX analysis were too general, based on the protein names and similarity percentage to the proteins in the NCBI database. Therefore, distinct protein classification analysis was performed to classify the proteins specifically, according to the Gene Ontology (GO) such as molecular function, biological processes and cell components. This approach was performed because the ontologies in GO are usually well controlled, structured and elucidated the function and products of the gene.

A total of nine sequence fragments was successfully annotated (sequence with annotation) while the other five sequence fragments were not identified (sequence without annotation) (Figure 1). Among the five fragments, two fragments (DEG3 and DEG5) showed sequence similarity with that of the NCBI database using a BlastX analysis (sequence with BLAST hits). On the other hand, three other fragments (DEG1, DEG2 and DEG4) did not present any similarity to the sequences from the database (sequence without BLAST hits).

Table 1
Total differential display cDNA fragments that were identified in samples of Cayratia trifolia *of soil and sludge. U: up-regulated fragment, D: down-regulated fragment*

Arbitrary primer (ACP)	Number of fragments	Name of fragments	Estimated size (bp)
ACP 1	1	DEG1 (U)	200
ACP 2	1	DEG2 (U)	300
ACP 3	3	DEG3 (U), DEG4 (U), DEG5 (U)	500 400 200
ACP 4	1	DEG6 (D)	700
ACP 5	1	DEG7 (U)	400
ACP 7	2	DEG8 (D), DEG9 (U)	400 500
ACP 8	2	DEG10 (U), DEG11(U)	700 300
ACP 9	3	DEG12 (U), DEG13 (U), DEG14 (U)	500 400 200
ACP 14	3	DEG15 (D), DEG 16 (U), DEG17 (U)	400 900 500
ACP 16	1	DEG18 (D)	600
ACP 17	2	DEG19 (D), DEG20 (U)	400 700
ACP 18	2	DEG21 (U), DEG22 (U)	800 300
ACP 19	1	DEG23 (U)	400

Table 2
BLASTX analyses of the DEGs amino acid sequence

Sequence name	Size (bp)	Similarity (%)	E-value	Description	Function	References	Organism	Accession number
DEG1	304	-	-	No similarity	-		-	-
DEG2	296	-	-	No similarity	-		-	-
DEG3	575	79	$4.76e^{-16}$	Broad-complex, Tramtrack and Bric-à-brac/poxvirus and zinc finger (BTB/POZ) domain-containing NPY5	Involved in plant organogenesis Play in root gravitropic responses	Cheng et al., 2008; Li et al., 2011	*Vitis vinifera*	XP_002284345.1
DEG4	393	-	-	No similarity	-		-	-
DEG5	274	83	$1.33e^{-10}$	Chlororespiratory reduction 42	Involved in respiration, cyclic electron transporters of Photosystem I (PSI) and carbon dioxide adoption	Munshi et al., 2005	*Pelargonium incrassatum*	AKH05251.1
DEG6	703	89	$2.9e^{-60}$	Metal transporter; natural resistance-associated macrophage protein (Nramp)	Involved in the uptake, translocation, intracellular transport, and detoxification of transition metals	Singh et al., 2016	*Methysia notabilis*	XP_010112784.1
DEG7	348	89	$1.71e^{-30}$	Protochlorophyllide-dependent translocon component 52	Involved in response to stresses	Sjuts et al., 2017	*Vitis vinifera*	XP_002283592.2
DEG8	368	93	$2.83e^{-37}$	Ribosomal L33 (chloroplast)	Involved in translation process	Ehrnthaler et al.,2014	*Tetrastigma hemsleyanum*	YP_009231268.1
DEG9	434	99	$6.76e^{-16}$	Haloacid dehalogenase-like hydrolase domain	Involved in various cellular processes	Koonin & Tatusov, 1994)	*Medicago truncatula*	XP_003625047.1
DEG13	324	83	$5.76e^{-26}$	SH3 domain-containing 2 isoform X1	Responsible for controlling protein-protein interactions in the signal transduction pathways	Zhuang & Jiang, 2015	*Vitis vinifera*	XP_002279562.1
DEG14	297	79	$9.7e^{-11}$	Suppressor of MAX2 1 isoform X2	Function in seed germination control	Stanga et al., 2013	*Vitis vinifera*	XP_010656698.1
DEG16	924	99	$7.15e^{-169}$	Aminoglycoside 3'-phosphotransferase	An antibiotic group used in the realm of genetic engineering	Nurizzo et al., 2003	*Enterobacter hormaechei*	WP_058675408.1
DEG17	382	93	$5.46e^{-14}$	Chalcone synthase	Work on the synthesis of secondary metabolite series in plants, fungi and bacteria	Han et al., 2016	*Lonicera japonica*	AFJ44312.1
DEG19	406	56	$3.25e^{-20}$	Glutaredoxin domain-containing cysteine-rich protein 1	Involved in electron carrier activity	Bick et al., 1998	*Jatropha curcas*	XP_012086699.1

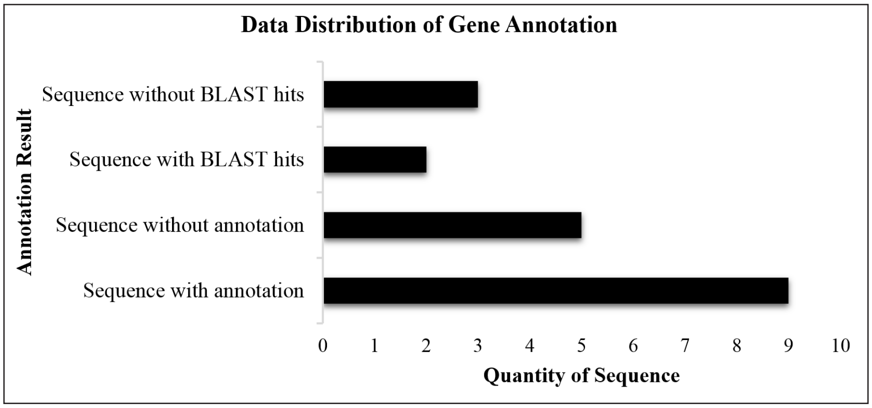

Figure 1. Distribution of the DEGs sequences annotation using Blast2GO

Subsequently, the protein sequences with predicted putative functions were classified according to the GO, which are molecular functions, biological processes and cellular components based on Blast2GO software. Molecular functions refer to the biochemical activities of the gene's product at a particular molecular level. While the biological processes indicate the biological objective of one or more of the said molecular functions. On the other hand, cellular components indicate the locations of the gene products before they become activated in the cell. The GO groups analyses have classified six protein sequences (DEG6, DEG7, DEG8, DEG9, DEG16 and DEG17) into the molecular functional group, while another six (DEG6, DEG7, DEG8, DEG14, DEG16 and DEG17) were classified into the biological process group and the others (DEG6, DEG7, DEG8 and DEG13) were in the cellular component group.

Our study prioritizes the GO distribution in the molecular functions category. It enables proteins functioning in response to environmental stress be identified, hence predict the genes that respond to the stresses. Four GO groups were identified based on their molecular functions such as catalytic activity (DEG7, DEG9, DEG16, DEG17), binding activity (DEG7, DEG 16), transporter activity (DEG6), and structural molecule activity (DEG8). The DEG7 sequence is classified both under the category of catalytic and binding activity. Both of these activities involved in plant stress response. Among these fragments, DEG7 fragment was identified and predicted as the cDNA encoding stress-responsive gene. This prediction was supported by the results from homology search using BlastX analyses and functional annotation and gene classification using Blast2GO.

Thus, DEG7 was selected for isolation and further classification. The information on DEG7 sequence was used for RACE-PCR and PCR analyses by using degenerated primer. Both the analyses resulted in the fragment with an estimated size of 1371 bp, named *CtSRG1* (*Cayratia trifolia Stress Responsive Gene*) gene (Accession no.: MG546210). BLAST was performed on

CtSRG1 and showed the highest similarity to the translocon (Table 3) that is important to the stress response. The presence of protein domain identified using Conserved Domain software NCBI revealed that CtSRG1 protein consisted of Rieske and START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC (SRPBCC) ligand-binding domain. Protein in SRPBCC domain has been reported to have an important role in biotic and abiotic stresses (Guo et al., 2017).

Quantitative real time PCR (qRT-PCR) analysis revealed that the expression of *CtSRG1* gene varied according to different treatment. Comparison of *CtSRG1* gene expression in *C. trifolia* leaves treated with Pb, diesel, NaCl, and wounding were relative to the expression of *CtSRG1* gene in control *C. trifolia* leaf (untreated). Relative expression for *CtSRG1* gene is up-regulated if the fold value is more than 1 (> 1). Whereas, the relative expression of the *CtSRG1* gene is down-regulated when the fold value is less than 1 (<1).

Relative expression for *CtSRG1* gene was significantly up-regulated ($p<0.05$) when treated with Pb at the concentration of 0.01 and 0.3 g/kg. However *CtSRG1* gene was down-regulated when *C. trifolia* treated with Pb at the concentration of 0.6 g/kg (Figure 2A). Figure 2B shows *CtSRG1* gene expression up-regulated significantly in leaves of *C. trifolia* when treated with 50 and 100 mM NaCl. However, *CtSRG1* gene was down-regulated under a higher concentration of NaCl of 300 mM. The results of the qRT-PCR analyses showed that the relative expression of *CtSRG1* gene was up-regulated when treated with 40g/kg of diesel. However, the results of the qRT-PCR analyses showed that the expression of *CtSRG1* gene was down-regulated under higher diesel treatment of 70 and 100 g/kg (Figure 2C). Overall, it is notable that the expression of *CtSRG1* gene is down-regulated when treated with wounding (Figure 2D).

DISCUSSION

In this study, DEG7 has the highest similarity to a stress responsive gene known as translocon. Translocon is a stress responsive protein complex that facilitate polypeptide translocation across cell membrane to balance cell homeostasis during environmental stress (Pogson et al., 2015; Sjuts et al., 2017; Yan et al., 2014). Plants such as *Arabidopsis thaliana* and *Solanum lycopersium* up-regulated their translocon gene expression during heat

Table 3
BLASTX analyses of the CtSRG1 amino acid sequence

Description	Organism	Accession No.	Similarity (%)	E-value
Translocon	*Vitis vinifera*	XP_002283592.2	67	$5e^{-173}$
ACD1-like protein	*Theobroma cacao*	EOY32295.1	54	$8e^{-148}$
Protein containing- Rieske domain (2Fe-2s)	*Populus trichocarpa*	XP_002299108.1	47	$3e^{-123}$
Pheophorbida a oxigenase	*Cynara cardunculus*	KVI08321.1	44	2^{e-117}

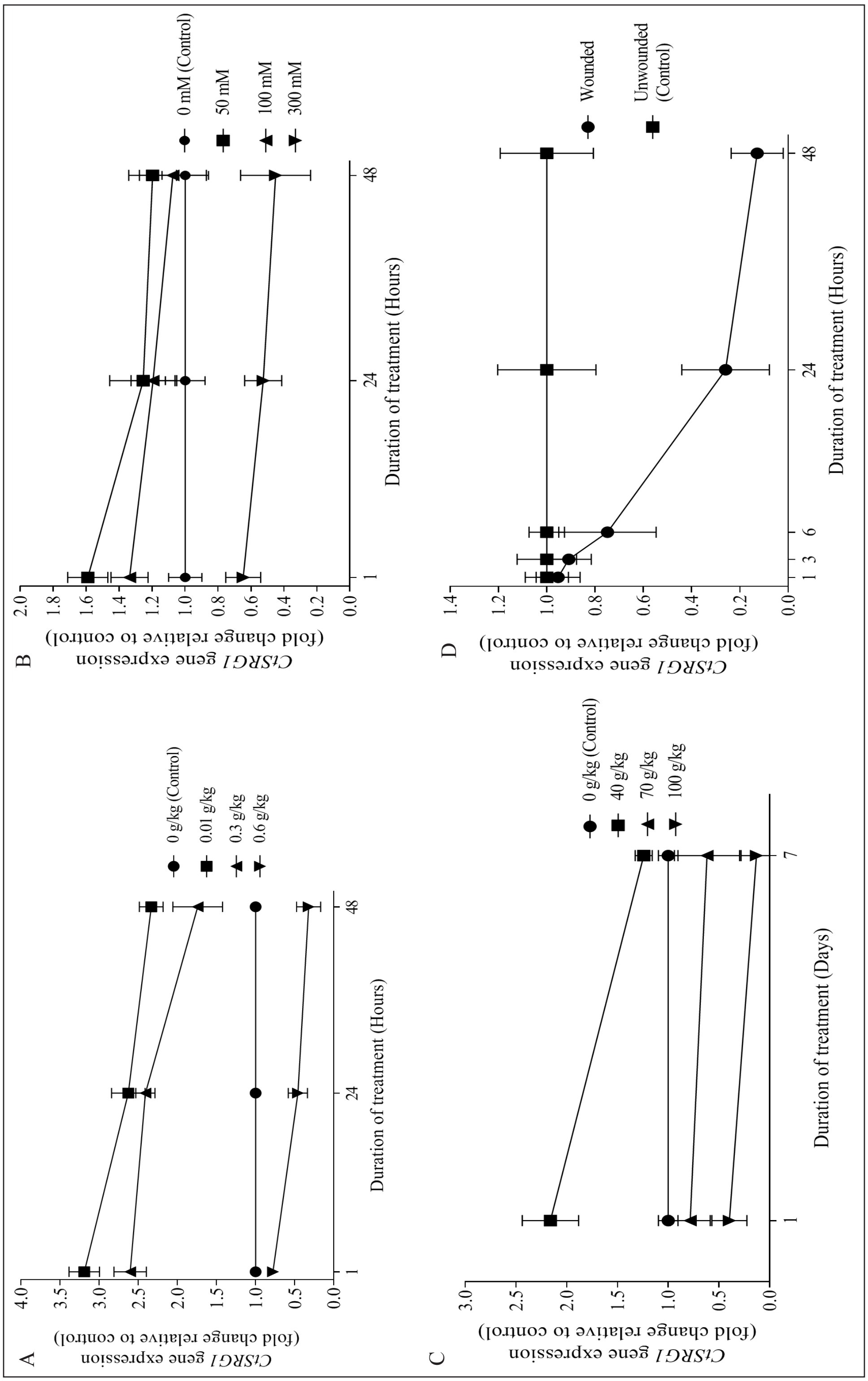

Figure 2. Changes in expression of the *CtSRG1* gene when treated with Pb (A), NaCl (B), diesel (C) and wounding (D). Vertical bars represent the means ± SD (n= 3)

(Ko et al., 2005) and NaCl stress (Yan et al., 2014), respectively. The DEG7 sequence is also classified under catalytic and binding activity category. Catalytic activity involving enzymes regulation is necessary for plant to maintain their cell homeostasis (Ryšlavá et al., 2013). Being a sessile organism, plant cannot avoid adverse effect of environmental stress. Thus, plant has to regulate specific enzyme activities to increase their survival rate (Doubnerova & Ryslava, 2013). On the other hand, binding activity also plays a role in plant survival against environmental stress. Protein binding is the center of the regulatory factors that control post-transcriptional RNA metabolism during the growth and development of plants as well as in response to stress (Lee & Kang, 2016).

CtSRG1 protein consists of two ligan-binding domains which are Rieske and START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC (SRPBCC). SRPBCC superfamily is known as Bet_v1 or steroidogenic acute regulatory protein (StAR)-related lipid transfer (START), and this superfamily consists of hydrophobic ligand-binding pocket (Schrick et al., 2014). Many studies have shown the involvement of START protein in generating response and tolerance towards environmental stresses. In *Arabidopsis thaliana*, transcription factors such as NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) family was identified to possess transmembrane domain and take part during stress response (Clercq et al., 2013). In addition, a novel *ITN1* (Sakamoto et al., 2008) and *NTHK1* gene (Zhou et al., 2005) which encoded for transmembrane protein were also involved in stress tolerance to salt in *A. thaliana*. In *Cicer arietinum* L., a gene known as *TM-START* was identified and characterised, in which the gene was up-regulated when the plant experienced water, salt, wounding and heat stresses (Satheesh et al., 2016). All of the studies show the activation of proteins when they bind to a ligand, followed by signal transduction towards stresses. Proteins act as a transcription factor which induces pathways or as an extensive component of a signal transduction pathway by taking part in protein-protein interactions responsible for environmental stress response. Therefore, the analysis of *CtSRG1* gene and CtSRG1 protein sequence support the suggestion that *CtSRG1* gene is a suitable stress response gene to be used in the next study.

The outcomes of this study showed that the response of *CtSRG1* gene towards Pb, NaCl, diesel and wounding stresses can be classified into two categories, i.e. positive and negative feedback response. *CtSRG1* gene exhibited positive feedback response, in which the gene was up-regulated when treated with Pb, NaCl and diesel. In contrast, *CtSRG1* gene was classified as a negative feedback response gene when the gene was down-regulated during wounding treatment. Both of these findings showed that *CtSRG1* gene was responsive to chemical stresses of Pb, NaCl and diesel and also wounding stress. Gene with positive feedback response, is an up-regulated gene that acts as defence mechanism and crucial for plant survival during stress (Richter et al., 2010). One of the defence mechanism is the activation of stress response gene that acts as a positive regulator in the adaptation and tolerance of plant towards stress. Genes in which the expression are up-regulated during stress shows that they are ready to repair the stress condition in the plant system (de Nadal et al., 2011). Hence, as a stress response gene, *CtSRG1* might be involved in the positive feedback response towards Pb, NaCl and diesel stresses, and possibly act as an intermediate in defence signals of *C. trifolia* plant.

Meanwhile, this study discovered that *CtSRG1* gene was also involved in the negative feedback towards wounding stress, where the *CtSRG1* gene was being down-regulated during the wounding treatment period starting from the first hour until the 48th hour. Down regulation of *CtSRG1* gene shows the defence mechanism of *C. trifolia* may require other components such as lignin, callose and phenolic compound that are able to suppress the gene expression. For instance, when a plant is under wounding stress, the cell wall thickens due to lignin and callose deposition (Denness et al., 2011). Synthesis of phenolic compound also happens during localised response when the plant is wounded. These factors contribute to the suppression and the expression of a gene. Our results were in line with the findings observed by Li et al. (2016), where *HyPRP1* gene in tomato was down-regulated under heat, drought, wounding, NaCl and abscisic acid hormone (ABA) stresses. Instead, these stresses up regulate other antioxidant genes. Moreover, *HyPRP1* gene in *N. benthamiana* functions as a negative feedback mechanism towards pathogens invasion by negatively regulates the expression of other genes such as the defence and antioxidant genes (Yeom et al., 2012). This suggests that *CtSRG1* gene is responsive through negative feedback under wounding treatment, hence, helps *C. trifolia* to maintain its survival in PPMSB sludge contaminated area.

This study shows that *C. trifolia* plant is different from other plants since it possesses higher tolerance to Pb, NaCl and diesel concentrations. Thus, *C. trifolia* plant is potential to be used as a phytoremediation agent and can be applied directly to the environment in the future. However, when the plant were subjected to higher concentration of Pb (0.6 g/kg), NaCl (300 mM) and diesel (70 g/kg dan 100 g/kg), the *CtSRG1* gene was down-regulated. The concentration of 0.6 g/kg of Pb, 300 mM NaCl and 70-100 g/kg diesel are therefore toxic to *C. trifolia* plant. It can cause cell death and cell system failure due to the changes and damages of DNA and protein of the plant cells (Xu et al., 2017). Damaged DNA can disrupt genes transcription, protein translation and DNA replication (Xu et al., 2017) which correlate with down regulation of gene expression. Down regulation of stress response gene due to higher amount of Pb and NaCl were already reported in several plants such as *S. Lycopersicum* (Perez et al., 2013) and *Cuminum cyminum* L. (Soleimani et al., 2017), respectively. On the other hand, there is still no report regarding the actual toxicity level for Pb, NaCl and diesel in *C. trifolia*. Therefore, the current study is the first to describe on *C. trifolia* limit against Pb, NaCl and diesel stresses.

CONCLUSION

We have isolated a new stress responsive gene from *C. trifolia* namely *CtSRG1*. *CtSRG1* expression was up-regulated by the Pb, NaCl and diesel but down-regulated by wounding, indicating the gene plays a very siginificant role towards stresses adaptation. This corresponds to the functionality of the gene that has a high similarity to translocon and possesses Rieske and SRPBCC domain involved in the response to biotic and abiotic stresses. In addition, *C. trifolia* plant has also been proven to have a high level of phytotoxic resistance to Pb, NaCl and diesel stress. Futher study needs to be conducted in the future to understand the specific manner of *CtSRG1* involvements in plant stress adaptation toward Pb, NaCl, diesel and wouding stresses.

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REFERENCES

Afzal, M., Yousaf, S., Reichenauer, T. G., Kuffner, M., & Sessitsch, A. (2011). Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. *Journal of Hazardous Materials*, *186*(2-3), 1568-1575.

Agarwal, P., Bhatt, V., Singh, R., Das, M., Sopory, S. K., & Chikara, J. (2013). Pathogenesis-related gene, *JcPR-$_{10a}$* from *Jatropha curcas* exhibit RNase and antifungal activity. *Molecular Biotechnology*, *54*(2), 412–425.

AL-Jobori, Kamil. M., Kadhim, A. K., & AL-Erjan, A. M. (2019). Effect of lead metal stress on antioxidant genes expression in leaves of sunflower (*Helianthus annuus* L. cv. Aqmar) plants. *Plant Archives*, *19*(1), 284-292.

Bick, J., Aslund, F., Chen, Y., & Leustek, T. (1998). Glutaredoxin function for the carboxyl-terminal domain of the plant-type 5’-adenylylsulfate reductase. *Proceedings of the National Academy of Sciences*, *95*(14), 8404–8409.

Cheng, Y., Qin, G., Dai, X., & Zhao, Y. (2008). *NPY* genes and AGC kinases define two key steps in auxin-mediated organogenesis in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *105*(52), 21017–21022.

Clercq, I. D., Vermeirssen, V., Aken, O. V., Vandepoele, K., Murcha, M. W., Law, S. R., ... Van Breusegema, F. (2013). The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. *The Plant Cell Preview*, *25*(9), 3472–3490.

de Nadal, E., Ammerer, G., & Posas, F. (2011). Controlling gene expression in response to stress. *Nature Reviews Genetic*, *12*(12), 833–845.

Denness, L., McKenna, J. F., Segonzac, C., Wormit, A., Madhou, P., Bennett, M., ... Hamann, T. (2011). Cell wall damage-induced lignin biosynthesis is regulated by a reactive oxygen species- and jasmonic acid-dependent process in *Arabidopsis*. *Plant Physiology*, *156*(3), 1364–1374.

Doubnerova, V., & Ryslava, H. (2013). Enzyme regulation during plant stress. *Biochemistry and Analytical Biochemistry*, *2*(3), 1–2.

Ehrnthaler, M., Scharff, L. B., Fleischmann, T. T., Hasse, C., Ruf, S., & Bock, R. (2014). Synthetic lethality in the tobacco plastid ribosome and its rescue at elevated growth temperatures. *The Plant Cell*, *26*(2), 765–776.

Gogosz, A. M., Bona, C., Santos, G. O., & Botosso, P. C. (2010). Germination and initial growth of *Campomanesia xanthocarpa* O. Berg. (Myrtaceae), in petroleum-contaminated soil and bioremediated soil. *Brazilian Journal of Biology*, *70*(4), 977–986.

Guo, M., Li, H., Li, L., Cheng, X., Gao, W., Xu, Y., … Liu, X. (2017). Comparative proteomic analysis of *Arabidopsis thaliana* roots between wild type and its salt tolerant mutant. *Journal of Plant Interactions*, *9*(1), 330–337.

Gupta, J., Kumar, D., & Gupta, A. (2012). Evaluation of gastric anti-ulcer activity of methanolic extract of *Cayratia trifolia* in experimental animals. *Asian Pacific Journal of Tropical Disease*, *2*(2), 99-102.

Han, Y., Ding, T., Su, B., & Jiang, H. (2016). Genome-wide identification, characterization and expression analysis of the chalcone synthase family in maize. *International Journal of Molecular Sciences*, *17*(161), 1–15.

Issoufi, I., Rhykerd, R. L., & Smiciklas, K. D. (2006). Seedling growth of agronomic crops in crude oil contaminated soil. *Journal of Agronomy and Crop Science*, *192*(4), 310-317.

Kirchmann, H., Borjesson, G., Katterer, T., & Cohen, Y. (2017). From agricultural use of sewage sludge to nutrient extraction: A soil science outlook. *Ambio*, *46*(2), 143–154.

Ko, K., Taylor, D., Argenton, P., Innes, J., Pedram, B., Seibert, F., ... Ko, Z. (2005). Evidence that the plastid translocon Tic40 components possess modulating capabilities. *The American Society for Biochemistry and Molecular Biology*, *280*(1), 215–224.

Koonin, E. V., & Tatusov, R. L. (1994). Computer analysis of bacterial haloacid dehalogenases defines a large superfamily of hydrolases with diverse specificity. *Journal of Molecular Biology*, *244*(1), 125–132.

Kumar, D., Gupta, J., Gupta, A., Kumar, S., & Arya, R. (2011). A review on chemical and biological properties of *Cayratia trifolia* Linn. (Vitaceae). *Pharmacognosy Reviews*, *5*(10), 184-188.

Lee, K., & Kang, H. (2016). Emerging roles of RNA-binding proteins in plant growth, development, and stress responses. *Molecules and Cells*, *39*(3), 179–185.

Li, J., Ouyang, B., Wang, T., Luo, Z., Yang, C., Li, H., ... Ye, Z. (2016). *HyPRP1* gene suppressed by multiple stresses plays a negative role in abiotic stress tolerance in tomato. *Frontiers in Plant Science*, *7*(967), 1–14.

Li, Y., Dai, X., Cheng, Y., & Zhao, Y. (2011). *NPY* genes play an essential role in root gravitropic responses in *Arabidopsis*. *Molecular Plant*, *4*(1), 171–179.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402–408.

Morales, S. G., Trejo-Téllez, L. I., Merino, F. C. G., Caldana, C., Espinosa-Victoria, D., & Cabrera, B. E. H. (2012). Growth, photosynthetic activity and potassium and sodium concentration in rice plants under salt stress. *Acta Scientiarum Agronomy*, *34*(3), 317–324.

Munshi, M. K., Kobayashi, Y., & Shikanai, T. (2005). Identification of a novel protein, CRR7, required for the stabilization of the chloroplast NAD(P)H dehydrogenase complex in *Arabidopsis*. *The Plant Journal*, *4*(6), 1036–1044.

Nurizzo, D., Shewry, S. C., Perlin, M. H., Brown, S. A., Dholakia, J. N., Fuchs, R. L., ... Smith, C. A. (2003). The crystal structure of aminoglycoside-30-phospho-transferase-IIa, an enzyme responsible for antibiotic resistance. *Journal of Molecular Biology*, *327*(2), 491–506.

Park, J. M., Park, C. J., Lee, S. B., Ham, B. K., Shin, R., & Paek, K. H. (2011). Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell*, *13*(5), 1035-1046.

Pen˜a-Castro, J. M., Barrera-Figueroa, B. E., Ferna´ndez-Linares, L., Ruiz-Medrano, R., & Xoconostle-Ca´zares, B. (2006). Isolation and identification of up-regulated genes in bermudagrass roots (*Cynodon dactylon* L.) grown under petroleum hydrocarbon stress. *Plant Science*, *170*(4), 724–731.

Perez, S., Ahmed, A. I. S., & Cabezas, D. (2013). Molecular and biochemical characterization of tomato (*Solanum lycopersicum* L.) plants cv. Micro-Tom under lead (Pb)-induced stress. *Biotecnología Aplicada*, *30*(3), 194–198.

Pogson, B. J., Ganguly, D., & Albrecht-Borth, V. (2015). Insights into chloroplast biogenesis and development. *Biochimica et Biophysica Acta*, *1847*(9), 1017–1024.

Ragasa, C. Y., Buluran, A. I., Mandia, E. H., & Shen, C. C. (2014). Chemical constituents of *Cayratia trifolia*. *Der Pharma Chemica*, *6*(6), 418-422.

Richter, K., Haslbeck, M., & Buchner, J. (2010). The heat shock response: Life on the verge of death. *Molecular Cell*, *40*(2), 253–266.

Ryšlavá, H., Doubnerova, V., Kavan, D., & Vaněk, O. (2013). Effect of posttranslational modifications on enzyme function and assembly. *Journal of Proteomics*, *92*, 80-109.

Sakamoto, H., Matsuda, O., & Iba, K. (2008). *ITN1*, a novel gene encoding an ankyrin-repeat protein that affects the ABA-mediated production of reactive oxygen species and is involved in salt-stress tolerance in *Arabidopsis thaliana*. *The Plant Journal*, *56*(3), 411–422.

Sangeetha, J., & Thangadurai, D. (2014). Effect of biologically treated petroleum sludge on seed germination and seedling growth of *Vigna unguiculata* (L.) Walp. (Fabaceae). *Brazilian Archives of Biology and Technology*, *57*(3), 427–433.

Satheesh, V., Chidambaranathan, P., Jagannadham, P. T., Kumar, V., Jain, P. K., Chinnusamy, V., ... Srinivasan, R. (2016). Transmembrane START domain proteins: *In silico* identification, characterization and expression analysis under stress conditions in chickpea (*Cicer arietinum* L.). *Plant Signaling and Behavior*, *11*(2), 1–13.

Schrick, K., Bruno, M., Khosla, A., Cox, P. N., Marlatt, S. A., Roque, R. A., ... Yadav, G. (2014). Shared functions of plant and mammalian StAR-related lipid transfer (START) domains in modulating transcription factor activity. *BMC Biology*, *12*(70), 1–20.

Shavrukov, Y. (2012). Salt stress or salt shock: Which genes are we studying?. *Journal of Experimental Botany*, *64*(1), 119-127.

Singh, S., Mann, R., & Sharma, S. K. (2012). Phytochemical analysis and pharmacognostical standardization of stem of *Cayratia trifolia* (Linn.) Domin. *International Journal of Pharmaceutical Sciences and Research*, *3*(11), 4503–4506.

Singh, S., Parihar, P., Singh, R., Singh, V. P., & Prasad, S. M. (2016). Heavy metal tolerance in plants: Role of transcriptomics, proteomics, metabolomics, and ionomics. *Frontiers in Plant Science*, *6*(1143), 1–36.

Sjuts, I., Soll, J., & Bölter, B. (2017). Import of soluble proteins into chloroplasts and potential regulatory mechanisms. *Frontiers in Plant Science*, *8*(168), 1–15.

Soleimani, Z., Afshar, A. S., & Nematpour, F. S. (2017). Responses of antioxidant gene and enzymes to salinity stress in the *Cuminum cyminum* L. *Russian Journal of Plant Physiology*, *64*(3), 361–367.

Stanga, J. P., Smith, S. M., Briggs, W. R., & Nelson, D. C. (2013). *SUPPRESSOR OF MORE AXILLARY GROWTH2 1* controls seed germination and seedling development in *Arabidopsis*. *Plant Physiology*, *163*(1), 318–330.

Xu, F., Fang, Y., Yan, L., Xu, L., Zhang, S., Cao, Y., ... Wang, J. (2017). Nuclear localization of Beclin 1 promotes radiation-induced DNA damage repair independent of autophagy. *Scientific Reports*, *7*(45385), 1–11.

Yan, J., Campbell, J. H., Glick, B. R., Smith, M. D., & Liang, Y. (2014). Molecular characterization and expression analysis of chloroplast protein import components in tomato (*Solanum lycopersicum*). *PLoS One*, *9*(4), 1–13.

Yazid, R. M., & Sidik, N. M. (2011). Extraction of functional total RNA from *Cayratia trifolia*. *Advances in Environmental Biology*, *5*(10), 3404–3409.

Yeom, S. I., Seo, E., Oh, S. K., Kim, K. W., & Choi, D. (2012). A common plant cell-wall protein HyPRP1 has dual roles as a positive regulator of cell death and a negative regulator of basal defense against pathogens. *Plant Journals*, *69*(5), 755–768.

Zhou, H. L., Cao, W. H., Cao, Y. R., Liu, J., Hao, Y. J., Zhang, J. S., & Chen, S. Y. (2005). Roles of ethylene receptor NTHK1 domains in plant growth, stress response and protein phosphorylation. *FEBS Letters*, *580*(5), 1239–1250.

Zhuang, X., & Jiang, L. (2015). SH domain proteins in plants: Roles in signaling transduction and membrane trafficking. In N. Kurochkina (Ed.), *SH domains* (pp. 17–33). Basel, Switzerland: Springer.

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Review article

Genetic Approaches and Nutrient Management in Rice Soil: A Review

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ABSTRACT

Rice is a great source of energy and protein for the human population, and it is beneficial to human health. Also, rice is the staple food for more than half of the world's population. Micronutrients play an important role in increasing agricultural productivity, and various factors, including toxic levels of elements such as aluminum, iron, heavy metals and salts as well as deficiency of nutrients such as zinc and phosphorus, have negative effects on rice productivity. Deficiency of nutrients is becoming more serious due to increased nutrient demand from intensive agriculture. Different methods of applying nutrients have been suggested, but the application of fertilizers is unaffordable for many poor farmers in developing regions. There is considerable diversity in rice germplasm, and this variation can be exploited to develop tolerant rice varieties that can withstand unfavorable conditions through breeding programs. Regarding our review of problem soils, beside field nutrition management, many quantitative trait loci (QTLs) and gene specific markers have been determined and could be used in marker-aided breeding program. Moreover, a combination of genetic and agronomic strategies, such as the use of tolerant varieties, nutrient, soil and cultural management, may be more effective in attaining sustainable agriculture.

Keywords: Breeding, genetic improvement, micronutrient, nutrient management

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INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple food, and more than half of the world's population feed on it. It is cultivated in more than hundred countries, mostly in Asia. Rice respectively supplies 21 and 15 percent of the energy and protein needs of humans worldwide. It has been estimated that the world population will increase to about ten billion by the end of the current century; thus, an increase in the production of rice per unit area is critical (Depar et al., 2011; Farooq et al., 2018; Lal, 2009; Rehman et al., 2012).

Nowadays, agricultural inputs including pesticides, irrigation, seeds and fertilizers are applied, but sometimes, the yield is different across the field at the end of the cultivating season. Variations in soil texture, subsoil characteristics, organic matter, water holding capacity and salinity are all factors that may affect plant performance. Presumably, it is economical to add different amounts of agricultural inputs to parts of the field with different soils (Chan et al., 2006). Problem soils, characterized by deficiency of essential plant nutrients and mineral toxicity, are widespread worldwide, and they seriously affect the production of rice. These problem soils make up a significant proportion of rice production zones, which are invariably affiliated with poverty owing to low and unstable productivity (Ismail et al., 2007). Moreover, balanced application of micro and macronutrients is essential for filling the yield gap. Apart from nitrogen, phosphorus and potassium, zinc (Zn) deficiency, which is prevalent, also has a great impact on rice yield (Fageria et al., 2002; Quijano-Guerta et al., 2002). Generally, in addition to intense toxicity of salts and elements, such as aluminum, heavy metals, and iron, deficiency of other essential nutrients, including zinc, iron, and phosphorus, has negative effects on agricultural productivity (Ismail & Thomson, 2011). Farmers only add micronutrients once deficiency symptoms appear, but micronutrients' deficiency reduce yield before the appearance of symptoms (Das, 2014). Deficiency of micronutrients has become a major nutritional problem affecting many people in developing countries.

The term "micronutrient" does not mean that they are less important to plants than other nutrients. The growth and development of plants may be delayed if any of these elements is missing in the soil or is not suitable balanced with other nutrients. Two sources of available micronutrients exist in the soil: nutrients that are adsorbed onto soil colloids and nutrients in the form of salts that are dissolved in soil solution. In addition, a secondary momentous source of some micronutrients is organic matter. Most micronutrients are held strongly in complex natural mixes and are not promptly accessible to plants. However, they might be a pivotal source of micronutrients once they change to a form that is available to plants as organic matter disintegrates (Das, 2014). Rice is capable of accumulating high levels of metals, including Fe, Al, and Zn. These metals exist naturally in very small amounts in paddy soils, but acidity or concentration of phytotoxic ions is increased by long-term use of chemical fertilizers (Meng et al., 2017). The deficiency of many abiotic stress tolerance elements, such as P and Zn, as well as Fe and Al toxicities show overlapping and complicated characteristics, and this multifaceted nature had unfavorable impact on past breeding endeavors to achieve high yielding varieties by satisfactory adaptation to such stress conditions (Ismail et al., 2007). Hydroponic systems are an effective approach to test metal tolerance because environmental factors are well controlled (Marmiroli et al., 2011). Moreover, leaf color, growth rate, and the expanse of plant injury are measured to determine the metal sensitivity of the seedling (Audebert & Fofana, 2009; Meng et al., 2017).

These challenges force researchers to seek novel approaches to make appreciable progress in adaptation to deficiency and toxicity of minerals in rice growing area. Recent improvements in breeding methods, such as genomics and molecular marker techniques, as well as powerful phenotyping approaches have made it conceivable that the hereditary determinants of complicated characteristics related to stress resistance can be detected and that these versatile attributes can be fused to achieve high yielding rice cultivars while maintaining their quality and good agronomic attributes. This review concentrates on nutritional deficiencies (P and Zn) and toxicities (Al and Fe) as the important problems of rice soil. We briefly discuss nutrition management, such as application methods and the source of fertilizer, in addition to recent understanding of the genetic bases of tolerance, development, and efforts to cope with the rice soil problems.

Aluminum Toxicity

Aluminum (Al) toxicity is a principal constraint in acidic soils, and it is widespread in areas of rice planting in tropical regions (Ismail et al., 2007). Aluminum dissolves in the soil solution to yield Al^{3+}. In intense acidic soil condition, Al^{3+} is highly phytotoxic, and it quickly affects root growth, ultimately causing stunted root system and influencing the capability of a plant to uptake both nutrients and water (Famoso et al., 2011).

Some studies have illustrated the differences in the levels of tolerance to Al toxicity between and within species. In addition, a high Al tolerance in both hydroponic and field conditions has been demonstrated in rice genotypes (Famoso et al., 2010; Foy, 1988; Kochian et al., 2005). Rice is approximately 6 to 10 times more aluminum tolerant than other cereals, and the genes involved in this tolerance have not been comprehensively studied. Considering its high level of Al tolerance and good genetic resources, rice is a good model for assessing the physiology and genetics of Al tolerance (Famoso et al., 2011). It has been reported that Al-exclusion mechanisms rather than internal detoxification would be beneficial in rice (Ma et al., 2002).

There is wide variation between varieties in terms of Al tolerance. Several quantitative trait loci (QTLs) have been identified using a population derived from sensitive IR64 and tolerant Azucena cross for shoot weight, leaf bronzing, and iron concentration (Wu et al., 1998). The use of hydroponic system for screening has experienced difficulty, owing to the quick reduction of the iron content in the solution culture. However, a screening approach that could recognize sensitive and tolerant varieties has been developed and used for more accurate genetic studies (Shimizu et al., 2005).

The Effort to Cope with Aluminum Toxicity. Rice (*Oryza sativa* L.) is less susceptible to Al toxicity than other cereals (Famoso et al., 2010; Ma, 2007). Also, there is a genotypic distinction between *japonica* and *indica* cultivars. Several transcriptional factors have been identified regarding aluminum tolerance in rice, including Al resistance transcription factor 1 (*ART1*), Nramp aluminum transporter 1 (*Nrat1*), stress and ripening 5 (*ASR5*), and *WRKY22* (Arenhart et al., 2014; Li et al., 2018; Yamaji et al., 2009). All genes that are responsive to Al toxicity have essential tasks to carry out towards plant Al tolerance. *ART1* regulates the external and internal detoxification of Al by influencing about 30 genes (Ma et al., 2014; Yamaji et al., 2009). *Nrat1* facilitates the transportation of trivalent Al into root cells. Diminished Al uptake, enhanced Al binding to the cell wall, and enhanced Al effects are the results of *Nrat1* silencing. The genotypic distinction in Al tolerance in rice may be incompletely clarified by diverse expression of *Nrat1* (Xia et al., 2014).

Aluminum tolerance is a complex trait governed by various genes/QTLs in rice. *OsALS1* is a single-copy gene in the rice genome, and it plays a major role in Al tolerance in rice. It encodes a transporter that is possibly involved in detoxification through transport and Al aggregation into cell vacuoles (Huang et al., 2012; Simoes et al., 2012). In general, 148 QTLs were reported for Al tolerance in rice using linkage mapping by biparental crosses (Ma et al., 2002, 2009; Mao et al., 2004; Nguyen et al., 2001, 2002, 2003; Wu et al., 2000; Xue et al., 2006, 2007) and association mapping using natural populations (Famoso et al., 2011; Zhang et al., 2016). Al tolerant QTLs include a large-effect *ART1* located on chromosome 12 with LOD = 7.85 and R2 = 19.3% using a RIL population. In addition, three genomic regions, encompassing *STAR2*, *ART1*, and *Nrat1*, related to induced Al-sensitivity of rice mutants were detected using biparental mapping population (Famoso et al., 2011). A genome wide association study for relative root elongation was conducted by a diverse panel consisting of 150 rice landraces, and PSM365 explained the meaningful associations (20.03%) located at 21.4 Mb on chromosome 11 (Zhang et al., 2016).

Recently, multi-parent advanced generation inter-cross (MAGIC) populations were mapped using a 55 K rice SNP array and phenotype at the seedling stage for Zn, Fe and Al under a hydroponic system. A total of 30, 21, and 21 QTLs were detected for Zn, Fe and Al toxicity tolerance respectively. For multi tolerance of Fe, Zn and Al affiliated traits, QTLs have been identified in three genomic regions, MT3.2 on chromosome 3 (35.4-36.2Mb), MT1.2 on chromosome 1 (35.4–36.3Mb), and MT1.1 on chromosome 1 (35.4–36.3Mb). The chromosomal regions MT2.3 on chromosome 2 (30.5–31.6Mb), MT3.1 on chromosome 3 (12.5–12.8Mb), and MT6 on chromosome 6 (2.0–3.0Mb) possess QTLs for Zn and Al tolerance. The QTLs (MT9.1) for Fe and Al tolerance are located on chromosome 9 (14.2–14.7Mb) (Meng et al., 2017).

Iron Toxicity

Iron (Fe) is a fundamental microelement that manages distinctive essential mechanisms in plants. Through the redox status modification among the ferrous (Fe^{2+}) and ferric (Fe^{3+}) shape, Fe acts as an electron acceptor or donor, which is essential in the mechanisms involved in photosynthesis and respiration (Kobayashi & Nishizawa, 2012; Zhai et al., 2014). In addition, Fe acts as a co-factor of numerous enzymes (Briat & Lobreaux, 1997; Wu et al., 2017).

Although it is an important plant nutrient, excess Fe can cause toxicity in plant (Wu et al., 2014). Iron toxicity occurs once a large amount of Fe (II) accumulates in the soil; also, rainfall may cause accumulation by interflow in the lower slopes (Ponnamperuma, 1972). Iron toxicity in rice plant takes the form of toxic accumulation of Fe in leaves. It is related to excess concentration of Fe (II) in the soil solution (Ponnamperuma et al., 1955).

Iron deficiency causes chlorosis in the leaf veins, and deficiency symptom appears initially in the plant's young leaves (Das, 2014). Iron toxicity leads to oxidative stress through Fenton reaction (Wu et al., 2017). Due to toxicity of Fe, rice yield usually experiences losses of 12-100% (Sahrawat, 2004). Leaf bronzing in rice is one of the visible Fe toxicity symptoms, and it is accompanied by decreased root and shoot growth (Wu et al., 2014). Discoloration of leaf and leaf bronzing index (LBI) are used for evaluating the degree of Zn toxicity (Frei et al., 2010; Holler et al., 2014; Meng et al., 2017; Wu et al., 2014).

Tolerance Mechanisms and Interaction between Iron and Other Nutrients. Excess iron concentration in the soil solution may cause nutrient imbalance via its antagonistic impact on the acquisition of nutrients, including K, P, Mn and Zn (Fageria et al., 2008; Sahrawat, 2004, 2008, 2010). Various mechanisms of tolerance to Fe toxicity are recognized, such as root-based tolerance and shoot based tolerance. Root based mechanisms involve using a physical barrier that prevents excess iron absorption (Becker & Asch, 2005; Wu et al., 2014, 2017). Shoot based tolerance mechanisms involve storing iron in tissues that are less active in photosynthesis, such as the stem (Engel et al., 2012). Inside the cells, extra Fe could be stored in vacuoles to prevent stress (Moore et al., 2014). Furthermore, plastids were indicated to play a role in Fe toxicity tolerance in rice thorough their ability to hold up to 4000 Fe atoms (Briat et al., 2010).

Tanaka et al. (1966) reported that the Fe concentration in the culture solution that led to toxicity of Fe was lower at the vegetative growth stage of rice compared with the later growth stages. Cultural practices such as ridge planting, planting date, pre-submergence of soil and water management could be manipulated to decrease Fe toxicity in rice. Using Fe toxicity-tolerant rice cultivars is the most cost effective method. Therefore, a combination of tolerant cultivar and developed cultural practices can produce great results in high Fe toxicity conditions (Sahrawat, 2004; Sahrawat et al., 1996).

To cope with the constraints of different tolerance rankings, a good understanding of the adaptation mechanisms to various Fe toxic environments is required. Also, the physiological and genetic factors underlying those mechanisms must be considered. Rice varieties vary in their iron toxicity tolerance. The screening of rice varieties to determine those with better iron tolerance is a momentous component of research for decreasing iron toxicity. Genetic variations in adaptation and tolerance to iron toxic soils have been exploited for improving rice varieties with iron toxicity tolerance (Balasubramanian et al., 2007; Nozoe et al., 2008; Sahrawat, 2004, 2010; Sahrawat & Sika, 2002).

Mechanisms of tolerance were studied in intolerant (IR29) and the tolerant RIL (FL483) at seedling stage. In shoots, ascorbate oxidase and glutathione S-transferase genes demonstrated genotypic differences, and FL483 had higher ascorbate oxidase activity and lower dehydroascorbate reductase (Wu et al., 2017). Nayak et al. (2008) conducted a study on the iron tolerance of 65 genotypes in the field and indicated that they exhibited a wide range of tolerance to iron toxicity, and iron tolerant rice genotypes produced higher grain yields than the iron-susceptible cultivars in the respective duration groups (Nayak et al., 2008).

The Effort to Cope with Iron Toxicity. Several genetic studies investigate issues related to Fe toxicity tolerance, such as genes that are associated with iron transport, including *OsIRT1* (Lee & An, 2009), *OsNRAMP1* and *OsNRAMP2* (Zhou & Yang, 2004); storage proteins, such as ferritin (*OsFER*) (Stein et al., 2009); and transcription factor, such as *OsWRKY80* (Ricachenevsky et al., 2010). Several transporters that play a role in metal transportation in rice have been determined (Kobayashi & Nishizawa, 2012).

Multiple QTLs were localized at 36.8-41Mb on chromosome 1 (Wu et al., 2014). FL483, an inbred line that bears the QTLs *q*FETOX-1-2 and *q*FETOX-1-1 in this region, demonstrated less leaf bronzing notwithstanding similar shoot Fe content in comparison to IR29, a sensitive parental line (Wu et al., 2014). Furthermore, a genome wide association study was carried out by Matthus et al. (2015) using 329 rice accessions. Meaningful markers related to leaf bronzing symptoms were identified on chromosomes 1 and 5. The detected loci on chromosome 1 similarity localized with several QTLs had been previously identified in various studies (Dufey et al., 2015; Wu et al., 2014). Moreover, a total of 197 QTLs have been reported for Fe toxicity in rice (Dufey et al., 2009, 2015; Fukuda et al., 2012; Liu et al., 2016; Matthus et al., 2015; Ouyang et al., 2007; Ruengphayak et al., 2015; Shimizu et al., 2005; Wan et al., 2003, 2005; Wu et al., 1998, 2014; Zhang et al., 2013). Four chromosomal regions (CR) involved in Fe toxicity tolerance of rice have been identified between markers RM246-RM443, RM526-R758, C515-C25 and R1245-RM429 on chromosomes 1, 2, 3 and 7 respectively (Dufey et al., 2015).

Phosphorus Deficiency

Phosphorus (P) is another essential element for plant nutrition. Due to its bond dynamism and the propensity of some soils to fix it in various forms, it is sorely available for plant roots especially in most acid and alkaline soils (Ismail & Thomson, 2011). Deficiency of phosphorus is common in both lowland and upland rice growing regions. Phosphorus deficiency could lead to high sterility, growth reduction, poor grain quality, crop yield reduction, and developmental delays. However, P deficiency in about 50 percent of agricultural soils (Lynch, 2011) could be decreased by applying fertilizer, but for many poor farmers, this is not affordable. Further, excess phosphorus can be washed out from the soils, ultimately leading to water eutrophication (Ismail et al., 2007; Ismail & Thomson, 2011; Nestler & Wissuwa, 2016; Raven & Taylor, 2003).

Further, regarding better P-utilization efficiency in plants, a cost effective enhancement of crop yield in limited-phosphorus conditions could be obtained via improving P acquisition from the soil (Ismail et al., 2007). This could be performed either via improved phosphorus mobilization in the soil or enhanced soil exploration (Lynch, 2011; Nestler & Wissuwa, 2016; Richardson et al., 2009; Rose et al., 2013). Wissuwa and Ae (2001) reported an intense association between tolerance to P deficiency and root uptake efficiency / root size. A wide root system could be a trustworthy criterion for identification of P deficiency-tolerant genotypes. The capability of rice genotypes to solubilize phosphorus fixed in the soil has been reported (Ismail & Thomson, 2011; Lynch, 2007; Nestler & Wissuwa, 2016). There are various mechanisms for the remobilization of P, and they may involve the emission of organic acids with low molecular weight, such as citrate, in aerobic soil conditions (Kirk et al., 1999).

Plant Root Formation and Interaction between Phosphorus and Other Nutrients. Organic acids can act as chelating factors for iron and aluminum to free phosphorus in soil solution, and high rates of exclusion of P-solubilizing organic acid anions of roots was described in rice in response to P-deficiency (Ismail & Thomson, 2011). Moreover, deficiency of zinc leads to accumulation of P in plants (Welch & Norvell, 1993). Owing to Zn deficiency, plants may not be able to coordinate P absorption mechanism (Marschner & Cakmak, 1986). Iron and phosphorus have negative effects on Zn and decrease its uptake by the roots and its translocation to plant shoot (Prasad et al., 2016). Root hairs were indirectly and directly (Gahoonia & Nielsen, 1998) linked to P uptake from the soil, and a simulation model proposed that the length of root hairs is more important for P uptake (Zygalakis et al., 2011) than root hair density and longevity (Brown et al., 2012).

Recently, it was illustrated that a soil with P deficiency may lead to the formation of shorter root hairs in comparison with adequately P supplied soil (Nestler et al., 2016). The traditional variety DJ123 has high root efficiency, taking up 2.5-fold more P per cm^2 of root surface area in comparison to the modern variety IR64. The possible justification for the differences in root efficiency could be the differences in genotypes in terms of root hair formation. It was observed that the differences in root hair properties of rice genotypes were contingent on the growth media used. The development of denser and longer root hairs of rice was observed in nutrient solution with P deficiency, while the same response to P supply was not seen in the soil (Nestler et al., 2016).

The Effort to Cope with P Deficiency. Obtaining genotypes that are able to efficiently mine phosphorus will help in enhancing and sustaining yields in low input agricultural systems (Ismail & Thomson, 2011). Genotypic variation in P deficiency tolerance in rice have been reported, but breeding endeavors were focused on screening existing genotypes and advances in breeding varieties for better efficiency in soils with P deficiency more than improving genotypes to obtain those with higher P uptake efficiency (Fageria et al., 2002; Ismail et al., 2007). Landrace cultivars are more impressive in P acquisition than the modern genotypes (Wissuwa & Ae, 2001). These landraces are great sources for using donors to improve P deficiency tolerant varieties through conventional methods. Furthermore, they can serve as sources to detect QTLs association with important agronomical features (Ismail & Thomson, 2011). A mapping study on P deficiency tolerance in rice was done by Wissuwa et al. (1998) using a backcross inbred population derived from Nipponbare (sensitive) and Kasalath (tolerant) cross. Four QTLs for P uptake were identified on chromosomes 2, 6, 10, and 12, and the robust QTL on chromosome 12 controls most of the P deficiency tolerance variation. This P acquisition QTL explained about 28 percent of the phenotypic variation. Ni et al. (1998) used RILs from IR20 and IR55178-3B-9-3 cross and identified a similar major QTL at the same location. They determined P uptake efficiency as relative shoot dry weight, relative tillering ability, and relative root dry weight.

Recently, an identified rice cultivar 'Akamai' (Yamagata), obtained from *japonica* landrace collection, revealed high tolerance to low P availability (Dissanayaka et al., 2017). This high tolerance is achieved through two preferable physiological mechanisms, including remobilization of P from old leaves to young leaves and increased root growth in P deficiency. Quantitative trait loci mapping for P deficiency tolerance in rice revealed a major QTL associated with P deficiency tolerance (*Pup1*) on chromosome 12 (Wissuwa et al., 1998, 2002). Subsequently, the gene, recognized as phosphorus starvation tolerance 1 (*PSTOL1*), was identified as the causal gene of *Pup1* (Gamuyao et al., 2012; Heuer et al., 2009). *PSTOL1* involves a protein in phosphorus absorption due to the encoding of protein kinase, and it enhances early root growth. It is intriguing that this gene from genomes of *japonica* cultivars was lost during domestication process and remains functional in some other Asian cultivars (Vigueira et al., 2016). Recently, a large-effect gene was identified within *Pup1*, and the functional mechanism of this gene (named P-STarvation TOLerance 1, *OsPSTOL1*) had been revealed (Gamuyao et al., 2012).

In addition, an intermediate QTL located on chromosome 6 and several other minor QTLs were detected on several chromosomes (Ismail & Thomson, 2011). Another mapping study was conducted using mapping population derived by backcrossing a NIL-C443 possessing the QTL from tolerant donor parent to Nipponbare (Wissuwa & Ae, 2001). The identified QTL for P uptake (*Pup1*) has been fine mapped to a flanked marker on the long arm of chromosome 12 (Wissuwa, et al., 2002). Markers were also developed at the *Pup1* locus, which was used to transfer *Pup1* into some lowland and upland popular genotypes by MABC (Septiningsih et al., 2009). The region for root growth under P deficiency was recognized on the long arm of chromosome 6 using population derived from Kasalath (tolerant) and Gimbozu (intolerant) cross (Shimizu et al., 2004). The position of the *qREP-6* was determined using chromosome segment substitution lines (CSSLs) developed by the background of Nipponbare (Shimizu et al., 2008). The line carrying *qREP-6* had higher shoot phosphorus concentration and tillering ability in P deficiency condition. Also, this QTL was fine mapped using F2 population. It was suggested that this QTL had a high potential to be used in breeding programs targeting root traits for deficiency soils (Shimizu et al., 2008).

Zn Deficiency

Zinc is one of the essential micronutrients that are necessary for enzymes playing a role in lipids metabolism and a co-factor for more than 300 enzymes involved in plant metabolism (Hafeez et al., 2013; Sadeghzadeh, 2013). More than 30 percent of soils have Zn deficiency, which affects plant's growth and development (Hacisalihoglu & Kochian, 2003; Hafeez, et al., 2013; Rehman et al., 2012). The problem of Zn deficiency affects approximately 60–70 percent of people in Africa and Asia (Farooq et al., 2018). However, the frequency of Zn deficiency in rice is more than 50 percent compared with other crops faced with this problem. The low Zn concentration indirectly results in breeding for high yield and for pest and disease resistance. Thus, Zn deficiency is one of the main nutritional deficiencies that limit the production of rice (Farooq et al., 2018; Ghoneim, 2016; Rehman et al., 2012).

In general, Zn deficiency is envisaged in sandy soils, calcareous soils, peat soils, and soils containing high silicon and phosphorus (Hafeez et al., 2013). Symptoms of Zn deficiency become visible usually 2 to 3 weeks after transplanting, with expanding brown spots and veins in leaves that can wholly coat older leaves, smaller and chlorosis leaves, spikelet sterility, stunted growth, and death of the plant in some cases. However, plants that recover after 4–6 weeks will show considerable lag in maturity and decline in yield (Hafeez et al., 2013; Mustafa et al., 2011; Prasad et al., 2016). Zinc seems to affect the capacity for the absorption of water and transport in plants and decreases the negative impacts of short periods of salinity and heat stress (Hafeez et al., 2013). The chemical properties of soil, such as pH, organic matter, redox potential, pedogenic oxide and soil sulfur content, play an important role in regulating Zn solubility in soils (Alloway, 2009).

Nowadays, water deficiency has caused a shift towards water saving techniques in agriculture, from flooded to aerobic rice systems (Farooq et al., 2009, 2011). Notably, these water-saving approaches may decrease Zn availability (Gao et al., 2006). Zinc deficiency occurs in both conventional flooded (Farooq et al., 2018; Jan et al., 2015; Quijano-Guerta et al., 2002) and direct seeded aerobic rice production systems (Gao et al., 2006). Therefore, it is deduced that the submerged condition in which rice is mainly cultivated is the main cause of Zn deficiency because in this kind of condition, the potential of redox reduces and the formation of insoluble Zn compounds is enhanced, such as $Zn(OH)_2$ formed owing to increase in pH, $ZnCO_3$ formed because of the partial pressure of CO_2 and ZnS formed because of intense decrease in conditions (Jan et al., 2015). Zinc up taken by plants in the form of Zn^{2+} ion in the early stages of growth is extremely phytotoxic. It was observed that Zn^{2+} has an important role in photosynthesis. Particularly, it participates in the reduction of photosynthesis activity through the disintegration of chlorophylls in lichens (Meng et al., 2017; Rout & Das, 2003). The principal resistance mechanisms of Zn deficiency in plants are still not well comprehended. Multiple potential mechanisms have been suggested to increase tolerance of Zn deficiency: (1) increased availability of Zn in the soil for root uptake, (2) increased absorption of Zn by roots and translocation and re-translocation from old tissues, (3) cellular homeostasis to hold a higher Zn concentration in the cytoplasm, and (4) efficient use of Zn in active tissues and cells (Hacisalihoglu & Kochian, 2003).

Application and Interactions of Zinc with Other Nutrients. There are various methods of Zn fertilizer application with respect to rice, including soil application, foliar spray and seed treatment (Fageria et al., 2002; Farooq et al., 2018; Ghoneim, 2016; Johnson et al., 2005). Under conventional flooded production systems, soil application is the main method for Zn supply (Farooq et al., 2018; Rehman et al., 2012). Applying the appropriate Zn sources to the soil seems to be an appropriate tactic to increase the availability of Zn. In general, $ZnSO_4$ is the most widely applied Zn source due to its low cost and high solubility (Ghoneim, 2016), and compared with other sources (ZnO and Zn-EDTA), the application of $ZnSO_4$ is the best (Cakmak, 2008). Beside, $ZnSO_4$ is proposed as the best for seed preparation to construct the grain Zn focus in poor Zn soils as opposed to soil and foliar applications (Cakmak, 2008; Jan et al., 2015; Rengel et al., 1999; Yilmaz et al., 1997).

Different approaches of Zn application may produce different results in various rice production systems. For instance, soil application enhanced rice yields more than the use of foliar application of Zn in conventional flooded systems (Ghoneim, 2016). However, in dry seeded aerobic rice, the opposite was observed (Ghoneim, 2016; Ram et al., 2015). Khan et al. (2003) conducted a study on an alkaline calcareous soil and observed that each application approach increased paddy yield, but a higher increase was observed with soil application of Zn compared with foliar application and root dipping. Zinc application (10 kg/ha) on soil improved grain Zn concentration and grain yield in comparison with foliar application (Rana & Kashif, 2014).

Several studies have compared different Zn application methods, including soil, foliage and seed treatments with respect to rice (Farooq et al., 2018; Imran et al., 2015; Phattarakul et al., 2012). Farooq et al. (2018) examined the addition of Zn by foliar application, soil application, seed priming or seed coating, and they reported enhanced Zn concentration in grain and grain yield of both puddled transplanted flooded rice and dry seeded rice. There were slight differences in the yield under different application methods; accordingly, Zn concentration in the grain was least with seed coating and always highest or equal to the highest with soil application (Farooq et al., 2018). On the other hand, some researchers reported that foliar application of Zn was more efficient in comparison with soil application. This is because there are more chances for losses in soil application because of Zn adsorption and precipitation. Zinc application at the primary stages and panicle initiation is very important in rice. With foliar application, there are some possible problems, such as rainfall washing off the solution, quick drying of the spray solution, low penetration rate in thick leaf and incomplete translocation in the leaf of the plant (Jan et al., 2015). Arif et al. (2006) reported that foliar application of micronutrients enhanced the number of spikelet per spike and the 1000-kernel weight. Also, Ghani et al. (1990) and Naik and Das (2007) reported that soil application of zinc enhanced the 1000-kernel weight of rice. Zinc application on nursery had no meaningful effect on grain yield. Further, zinc solution sprayed on rice seedlings three weeks after transplanting was the most impressive post transplanting treatment that helped to overcome Zn deficiency. Foliar spray can be used effectively to cope with the problem of micronutrient deficiency in the sub-soil (Mustafa et al., 2011).

The application of nitrogen fertilizer demonstrated a negative effect on rice grain Zn-concentration; generally, enhanced nitrogen application adversely influences grain Zn (Kutman et al., 2010; Shi et al., 2010). The interaction between zinc and nitrogen has been shown to have a synergistic effect in rice (Lakshmanan et al., 2005). Kutman et al. (2011) reported that N increased Zn absorption via the roots and its translocation to the shoot as well. Nonetheless, high levels of nitrogen lead to extreme vegetative growth, which can induce Zn deficiency in plants growing on Zn deficient soils (Ozanne, 1955; Prasad et al., 2016).

Furthermore, the application of phosphorus fertilizer not only reduces exchangeable and water soluble Zn, but it also enhances bound Zn forms in the soil (Mandal & Mandal, 1990). Also, phosphorus fertilizer application reduces the Zn concentration in the root and shoot. Other studies on rice showed that P application affects Zn uptake and its translocation to the shoot (Chatterjee et al., 1982; Haldar & Mandal, 1981; Lal et al., 2000; Prasad et al., 2016; Rehman et al., 2012). Several macronutrients, including magnesium, calcium, sodium and potassium, are known to prevent Zn uptake via plant roots in solution culture experiments. However, it seems that in soil experiments, their major effect is on soil pH (Alloway, 2009). Haldar and Mandal (1981) reported that Zn application decreased the concentration of Fe and Cu, but it enhanced Mn accumulation in rice root and shoot. A depletion in Zn concentration owing to iron fertilization was demonstrated in rice (Prasad et al., 2016).

The Effort to Cope with Zn Deficiency. Deficiency of zinc could be amended via applying Zn compounds to the plant or soil, but it is expensive to add Zn fertilizers in adequate quantities to cope with Zn deficiency; thus, adding fertilizer is not affordable for poor farmers. It has, therefore, been recommended that breeding efforts be strengthened by researchers to develop Zn deficiency tolerance rice cultivars (Singh et al., 2005; Wissuwa et al., 2006). The existence of great genotypic diversity in terms of grain Zn concentration (Gregorio, 2002; Shi et al., 2009) and the different genotypic behaviors in response to Zn deficiency shows the possibility of breeding using conventional methods to develop high yielding rice varieties with suitable Zn concentration in the grain (Ismail et al., 2007; Wissuwa et al., 2008). It seems that the trait of high grain Zn is strongly connected with aroma (Gregorio, 2002; Welch & Graham, 2004). Several reports indicated a significant adverse correlation between yield and grain Zn concentration in rice (Jiang et al., 2008; Wissuwa et al., 2008), but a positive association between grain Zn concentration and grain yield was observed in Zn deficient soil (Gregorio, 2002). Also, in various panels of landraces and aromatic rice in Zn adequate condition, a non-significant relationship was observed between grain Zn and yield (Swamy et al., 2016).

Zinc-regulated transporter and iron-regulated transporter, such as proteins (ZIPs), generally contribute to metal-ion homeostasis by moving cations into the cytoplasm (Colangelo & Guerinot, 2006).

Rice wild relatives are great sources of grains with high Zn content. Wild species of rice, including *O. nivara*, *O. latifolia*, *O. rufipogon*, *O. granulate*, and *O. officinalis*, also possess higher amounts of Zn than cultivated rice (Anuradha et al., 2012; Banerjee et al., 2010). Gregorio (2002) reported that aromatic rice has high Zn in comparison with non-aromatic rice. It has also been reported that the amounts of Zn in three IR64 mutant genotypes of polished rice, including M-IR-180, M-IR-175, and M-IR-49, were more compared with IR64. These mutants could be exploited in breeding programs for Zn deficiency and in understanding Zn mechanisms (Swamy et al., 2016).

Genetic studies of rice genome revealed that rice has nine heavy metal ATPases (HMA) genes. Three of these genes, including *OsHMA1-3*, have important roles in transporting Zn (Miyadate et al., 2011).

Mapping quantitative trait loci (QTLs) for Zn deficiency tolerance is a useful method to cope with this constraint. By detecting QTLs related to symptoms of Zn deficiency, it is possible to analyze the entire Zn deficiency response of different genetic factors associated with tolerance mechanism (Wissuwa et al., 2006). A genome wide association mapping detected meaningful SNPs on chromosomes 3 and 9 with respect to grain Zn (Norton et al., 2014). Rice varieties, such as IR64, NSICRc222, BR29, Swarna, BR11, PSBRc82, Ciherang, BR28, and Swarna Sub1, improved at IRRI have high Zn material background (Swamy et al., 2016). Likewise, considering the analysis of 21 metal genes in 12 rice genotypes, 39 SSR markers and 179 novel SNPs were detected for grain Zn (Banerjee et al., 2010). Moreover, associations between various grain Zn traits and SSR markers have also been demonstrated in various rice populations and germplasm (Brar et al., 2015; Swamy et al., 2016). Several mapping populations have been utilized in mapping studies for grain Zn (Anuradha et al., 2012; Norton et al., 2010; Zhang et al., 2011).

The detected grain Zn QTLs on chromosomes 7, 11, and 12 are suitable targets for marker assisted selection program. Three studies have found 53 QTLs for Zn tolerance using RIL populations (Liu et al., 2016; Zhang et al., 2013). The robust QTL *q*ZNT-1 on chromosome 1 at marker interval *XNpb93*-C3029C justified 21.9 percent of phenotypic variance (Dong et al., 2006). It is obvious that high grain Zn QTLs are spread over the genome to co-locate with other mineral elements' QTLs for the grain. The region on chromosome 5 (*qSdw5*) at interval 17.3–19.5Mb (Zhang et al., 2013) and *qFRSDW11* on chromosome 11 between C11S49-C11S60 (Liu et al., 2016) were identified for Zn and Fe stresses. It seems that there is a genomic overlap in tolerance to Zn deficiency and Fe toxicity in rice.

CONCLUSION

Phosphorus and zinc deficiencies are the most faced deficiencies in rice soils, since they are in forms that are not easily available to the root of plants. Excess amounts of Fe and Al caused toxicity in some rice growing areas. There is great genetic diversity in the response to nutritional deficiencies and toxicities in rice germplasm, which could be applied to improve the tolerance of rice cultivars. Identifying the various bases for nutrient deficiency and metal toxicity will increase breeding efficiency. Since the genetic bases for deficiencies and toxicities are complicated and involve several small-effect QTLs and are seriously affected by the environment, the choice of suitable breeding approaches, crossing programs, screening methods and field evaluation processes are crucial for the improvement of tolerant rice varieties. This review of previous studies indicates that deficiency and toxicity could be decreased by combining the use of tolerant varieties, nutrient, soil and cultural management. This integration is more practical for enhancing sustainable rice productivity.

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REFERENCES

Alloway, B. J. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environmental Geochemistry and Health*, *31*(5), 537-548.

Anuradha, K., Agarwal, S., Batchu, A., Babu, A., Swamy, B., Longva, T., & Sarla, N. (2012). Evaluating rice germplasm for iron and zinc concentration in brown rice and seed dimensions. *Journal of Phytology*, *4*(1), 19-25.

Arenhart, R. A., Bai, Y., de Oliveira, L. F. V., Neto, L. B., Schunemann, M., dos Santos Maraschin, F., ... Wang, Z. Y. (2014). New insights into aluminum tolerance in rice: The ASR5 protein binds the *STAR1* promoter and other aluminum-responsive genes. *Molecular Plant*, *7*(4), 709-721.

Arif, M., Chohan, M. A., Ali, S., Gul, R., & Khan, S. (2006). Response of wheat to foliar application of nutrients. *Journal of Agricultural and Biological Science, 1*(4), 30-34.

Audebert, A., & Fofana, M. (2009). Rice yield gap due to iron toxicity in West Africa. *Journal of Agronomy and Crop Science*, *195*(1), 66-76.

Balasubramanian, V., Sie, M., Hijmans, R. J., & Otsuka, K. (2007). Increasing rice production in Sub-Saharan Africa: Challenges and opportunities. *Advances in Agronomy*, *94*, 55-133.

Banerjee, S., Sharma, D. J., Verulkar, S. B., & Chandel, G. (2010). Use of *in silico* and semi quantitative RT-PCR approaches to develop nutrient rich rice (*Oryza sativa* L.) India. *Indian Journal of Biotechnology*, *9*, 203-212.

Becker, M., & Asch, F. (2005). Iron toxicity in rice - Conditions and management concepts. *Journal of Plant Nutrition and Soil Science*, *168*(4), 558-573.

Brar, B., Jain, R. K., & Jain, S. (2015). Correlation of molecular marker allele size with physio-morphological and micronutrient (Zn, Fe) traits among rice genotypes. *International Journal of Current Science*, *15*, 42-50.

Briat, J. F., Ravet, K., Arnaud, N., Duc, C., Boucherez, J., Touraine, B., … Gaymard, F. (2010). New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. *Annals of Botany*, *105*(5), 811-822.

Briat, J., & Lobreaux, S. (1997). Iron transport and storage in plants. *Trends in Plant Science*, *2*(5), 187-193.

Brown, L. K., George, T. S., Thompson, J. A., Wright, G., Lyon, J., Dupuy, L., … White, P. J. (2012). What are the implications of variation in root hair length on tolerance to phosphorus deficiency in combination with water stress in barley (*Hordeum vulgare*)?. *Annals of Botany*, *110*(2), 319-328.

Cakmak, I. (2008). Enrichment of cereal grains with zinc: Agronomic or genetic biofortification. *Plant and Soil*, *302*(1-2), 1-17.

Chan, C. S., Amin, M. S. M., Lee, T. S., & Mohammud, C. H. (2006). Predicting paddy soil productivity. *The Institution of Engineers, Malaysia*, *67*(4), 45-55.

Chatterjee, A. K., Mandal, L. N., & Haldar, M. (1982). Interaction of zinc and phosphorus in relation to micronutrient nutrition of rice plant at two different growth stages. *Journal of Plant Nutrition and Soil Science*, *145*(5), 460-469.

Colangelo, E. P., & Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Current Opinion in Plant Biology*, *9*(3), 322-330.

Das, S. K. (2014). Role of micronutrient in rice cultivation and management strategy in organic agriculture — A reappraisal. *Agricultural Sciences*, *5*(July), 765-769.

Depar, N., Rajpar, I., Memon, M. Y., & Imtiaz, M. (2011). Mineral nutrient densities in some domestic and exotic rice genotypes. *Pakistan Journal of Agriculture: Agricultural Engineering Veterinary Sciences*, *27*(2), 134-142.

Dissanayaka, D. M. S. B., Maruyama, H., Nishida, S., Tawaraya, K., & Wasaki, J. (2017). Landrace of *japonica* rice, Akamai exhibits enhanced root growth and efficient leaf phosphorus remobilization in response to limited phosphorus availability. *Plant and Soil*, *414*(1-2), 327-338.

Dong, Y., Ogawa, T., Lin, D., Koh, H. J., Kamiunten, H., Matsuo, M., & Cheng, S. (2006). Molecular mapping of quantitative trait loci for zinc toxicity tolerance in rice seedling (*Oryza sativa* L.). *Field Crops Research*, *95*(2-3), 420-425.

Dufey, I., Draye, X., Lutts, S., Lorieux, M., Martinez, C., & Bertin, P. (2015). Novel QTLs in an interspecific backcross *Oryza sativa* × *Oryza glaberrima* for resistance to iron toxicity in rice. *Euphytica*, *204*(3), 609-625.

Dufey, I., Hakizimana, P., Draye, X., Lutts, S., & Bertin, P. (2009). QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. *Euphytica*, *167*(2), 143-160.

Engel, K., Asch, F., & Becker, M. (2012). Classification of rice genotypes based on their mechanisms of adaptation to iron toxicity. *Journal of Plant Nutrition and Soil Science*, *175*(6), 871-881.

Fageria, N. K., Baligar, V. C., & Clark, R. B. (2002). Micronutrients in crop production. In *Advances in agronomy* (Volume 77, pp. 185-268). Cambridge, USA: Academic Press.

Fageria, N. K., Santos, A. B., Barbosa Filho, M. P., & Guimaraes, C. M. (2008). Iron toxicity in lowland rice. *Journal of Plant Nutrition*, *31*(9), 1676-1697.

Famoso, A. N., Clark, R. T., Shaff, J. E., Craft, E., McCouch, S. R., & Kochian, L. V. (2010). Development of a novel aluminum tolerance phenotyping platform used for comparisons of cereal aluminum tolerance and investigations into rice aluminum tolerance mechanisms. *Plant Physiology*, *153*(4), 1678-1691.

Famoso, A. N., Zhao, K., Clark, R. T., Tung, C. W., Wright, M. H., Bustamante, C., … McCouch, S. R. (2011). Genetic architecture of aluminum tolerance in rice (*Oryza sativa*) determined through genome-wide association analysis and QTL mapping. *PLOS Genetics*, *7*(8), e1002221.

Farooq, M., Aziz, T., & Habib, M. (2011). Boron nutripriming improves the germination and early seedling growth of rice (*Oryza Sativa* L.). *Journal of Plant Nutrition*, *34*(10), 1507-1515.

Farooq, M., Kobayashi, N., Wahid, A., Ito, O., & Basra, S. M. A. (2009). 6 Strategies for producing more rice with less water. *Advances in Agronomy*, *101*(4), 351-388.

Farooq, M., Ullah, A., Rehman, A., Nawaz, A., Nadeem, A., Wakeel, A., … Siddique, K. H. M. (2018). Application of zinc improves the productivity and biofortification of fine grain aromatic rice grown in dry seeded and puddled transplanted production systems. *Field Crops Research*, *216*, 53-62.

Foy, C. D. (1988). Plant adaptation to acid, aluminum-toxic soils. *Communications in Soil Science and Plant Analysis*, *19*(7-12), 959-987.

Frei, M., Tanaka, J. P., Chen, C. P., & Wissuwa, M. (2010). Mechanisms of ozone tolerance in rice: characterization of two QTLs affecting leaf bronzing by gene expression profiling and biochemical analyses. *Journal of Experimental Botany*, *61*(5), 1405-1417.

Fukuda, A., Shiratsuchi, H., Fukushima, A., Yamaguchi, H., Mochida, H., Terao, T., & Ogiwara, H. (2012). Detection of chromosomal regions affecting iron concentration in rice shoots subjected to excess ferrous iron using chromosomal segment substitution lines between *japonica* and *indica*. *Plant Production Science*, *15*(3), 183-191.

Gahoonia, T. S., & Nielsen, N. E. (1998). Direct evidence on participation of root hairs in phosphorus (^{32}P) uptake from soil. *Plant and Soil*, *198*(2), 147–152.

Gamuyao, R., Chin, J. H., Pariasca-Tanaka, J., Pesaresi, P., Catausan, S., Dalid, C., ... Heuer, S. (2012). The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency. *Nature*, *488*(7412), 535.

Gao, X., Zou, C., Fan, X., Zhang, F., & Hoffland, E. (2006). From flooded to aerobic conditions in rice cultivation: Consequences for zinc uptake. *Plant and Soil*, *280*(1-2), 41-47.

Ghani, A., Shah, M., & Khan, D. R. (1990). Response of rice to elevated rates of zinc in mountainous areas of Swat. *Sarhad Journal of Agriculture*, *6*(4), 411-415.

Ghoneim, M. (2016). Effect of different methods of Zn application on rice growth, yield and nutrients dynamics in plant and soil. *Journal of Agriculture and Ecology Research International*, *6*(2), 1-9.

Gregorio, G. B. (2002). Progress in breeding for trace minerals in staple crops. *The Journal of Nutrition*, *132*(3), 500S-502S.

Hacisalihoglu, G., & Kochian, L. V. (2003). How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytologist*, *159*(2), 341-350.

Hafeez, B., Khanif, M., & Saleem, M. (2013). Role of zinc in plant nutrition - A review. *American Journal of Experimental Agriculture*, *3*(2), 374-391.

Haldar, M., & Mandal, L. N. (1981). Effect of phosphorus and zinc on the growth and phosphorus, zinc, copper, iron and manganese nutrition of rice. *Plant and Soil*, *59*(3), 415-425.

Heuer, S., Lu, X., Chin, J. H., Pariasca-Tanaka, J., Kanamori, H., Matsumoto, T., … Wissuwa M. (2009). Comparative sequence analyses of the major QTL *p*hosphorus *up*take *1* (*Pup1*) reveal a complex genetic structure. *Plant Biotechnology Journal*, *7*(5), 456-471.

Holler, S., Hajirezaei, M. R., von Wiren, N., & Frei, M. (2014). Ascorbate metabolism in rice genotypes differing in zinc efficiency. *Planta*, *239*(2), 367-379.

Huang, C. F., Yamaji, N., Chen, Z., & Ma, J. F. (2012). A tonoplast-localized half-size ABC transporter is required for internal detoxification of aluminum in rice. *The Plant Journal*, *69*(5), 857-867.

Imran, M., Kanwal, S., Hussain, S., Aziz, T., & Maqsood, M. A. (2015). Efficacy of zinc application methods for concentration and estimated bioavailability of zinc in grains of rice grown on a calcareous soil. *Pakistan Journal of Agricultural Sciences*, *52*(1), 169-175.

Ismail A. M., & Thomson M. J. (2011). Molecular breeding of rice for problem soils. In A. Costa de Oliveira & R. Varshney (Eds.), *Root genomics* (pp. 289–311). Heidelberg, Germany: Springer.

Ismail, A. M., Heuer, S., Thomson, M. J., & Wissuwa, M. (2007). Genetic and genomic approaches to develop rice germplasm for problem soils. *Plant Molecular Biology*, *65*(4), 547-570.

Jan M., Anwar-ul-Haq M., Akhtar J., & Waraich E. A. (2015). Zinc application to rice genotypes under saline conditions. In E. Lichtfouse (Ed.), *Sustainable agriculture reviews* (pp. 253-272). Cham, Switzerland: Springer.

Jiang, W., Struik, P. C., van Keulen, H., Zhao, M., Jin, L. N., & Stomph, T. J. (2008). Does increased zinc uptake enhance grain zinc mass concentration in rice. *Annals of Applied Biology*, *153*(1), 135-147.

Johnson, S. E., Lauren, J. G., Welch, R. M., & Duxbury, J. M. (2005). A comparison of the effects of micronutrient seed priming and soil fertilization on the mineral nutrition of chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*) in Nepal. *Experimental Agriculture*, *41*(4), 427-448.

Khan, M. U., Qasim, M., Subhan, M., Jamil, M., & Ahmad, R. (2003). Response of rice to different methods of zinc application in calcareous soil. *Journal of Applied Sciences*, *3*(7), 524-529.

Kirk, G. J. D., Santos, E. E., & Santos, M. B. (1999). Phosphate solubilization by organic anion excretion from rice growing in aerobic soil: Rates of excretion and decomposition, effects on rhizosphere pH and effects on phosphate solubility and uptake. *New Phytologist*, *142*(2), 185-200.

Kobayashi, T., & Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Annual Review of Plant Biology*, *63*(1), 131-152.

Kochian, L. V, Piñeros, M. A., & Hoekenga, O. A. (2005). The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. *Plant and Soil*, *274*(1-2), 175-195.

Kutman, U. B., Yildiz, B., & Cakmak, I. (2011). Effect of nitrogen on uptake, remobilization and partitioning of zinc and iron throughout the development of durum wheat. *Plant and Soil*, *342*(1-2), 149-164.

Lakshmanan, R., Prasad, R., & Jain, M. (2005). Yield and uptake of micronutrients by rice as influenced by duration of variety and nitrogen fertilization. *Archives of Agronomy and Soil Science*, *51*(1), 1-14.

Lal, B., Majumdar, B., & Venkatesh, M. S. (2000). Individual and interactive effects of phosphorus and zinc in lowland rice. *Indian Journal of Hill Farming*, *13*(1-2), 44-46.

Lal, R. (2009). Laws of sustainable soil management. *Agronomy for Sustainable Development*, *29*(1), 7-9.

Lee, S., & An, G. (2009). Over-expression of *OsIRT1* leads to increased iron and zinc accumulations in rice. *Plant, Cell and Environment*, *32*(4), 408-416.

Li, G. Z., Wang, Z. Q., Yokosho, K., Ding, B., Fan, W., Gong, Q. Q., ... Zheng, S. J. (2018). Transcription factor WRKY 22 promotes aluminum tolerance via activation of *OsFRDL4* expression and enhancement of citrate secretion in rice (*Oryza sativa*). *New Phytologist*, *219*(1), 149-162.

Liu, H., Soomro, A., Zhu, Y., Qiu, X., Chen, K., Zheng, T., … Xu, J. (2016). QTL underlying iron and zinc toxicity tolerances at seedling stage revealed by two sets of reciprocal introgression populations of rice (*Oryza sativa* L.). *The Crop Journal*, *4*(4), 280-289.

Lynch, J. P. (2007). Roots of the second green revolution. *Australian Journal of Botany*, *55*(5), 493-512.

Lynch, J. P. (2011). Root phenes for enhanced soil exploration and phosphorus acquisition: Tools for future crops. *Plant Physiology*, *156*(3), 1041-1049.

Ma, J. F. (2007). Syndrome of aluminum toxicity and diversity of aluminum resistance in higher plants. *International Review of Cytology*, *264*, 225-252.

Ma, J. F., Chen, Z. C., & Shen, R. F. (2014). Molecular mechanisms of Al tolerance in gramineous plants. *Plant and Soil*, *381*(1-2), 1-12.

Ma, J. F., Shen, R., Zhao, Z., Wissuwa, M., Takeuchi, Y., Ebitani, T., & Yano, M. (2002). Response of rice to Al stress and identification of quantitative trait loci for Al tolerance. *Plant and Cell Physiology*, *43*(6), 652-659.

Ma, J., Huang, X., Wang, X., Chen, X., Qu, Z., Huang, L., & Kang, Z. (2009). Identification of expressed genes during compatible interaction between stripe rust (*Puccinia striiformis*) and wheat using a cDNA library. *BMC Genomics*, *10*(1), 586.

Mandal, B., & Mandal, L. N. (1990). Effect of phosphorus application on transformation of zinc fraction in soil and on the zinc nutrition of lowland rice. *Plant and Soil*, *121*(1), 115-123.

Mao, C., Yang, L., Zheng, B., Wu, Y., Liu, F., Yi, K., & Wu, P. (2004). Comparative mapping of QTLs for Al tolerance in rice and identification of positional Al-induced genes. *Journal of Zhejiang University Science*, *5*(6), 634-643.

Marmiroli, M., Pietrini, F., Maestri, E., Zacchini, M., Marmiroli, N., & Massacci, A. (2011). Growth, physiological and molecular traits in Salicaceae trees investigated for phytoremediation of heavy metals and organics. *Tree Physiology*, *31*(12), 1319-1334.

Marschner, H., & Cakmak, I. (1986). Mechanism of phosphorus-induced zinc deficiency in cotton. II. Evidence for impaired shoot control of phosphorus uptake and translocation under zinc deficiency. *Physiologia Plantarum*, *68*(3), 491-496.

Matthus, E., Wu, L. B., Ueda, Y., Holler, S., Becker, M., & Frei, M. (2015). Loci, genes, and mechanisms associated with tolerance to ferrous iron toxicity in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *128*(10), 2085-2098.

Meng, L., Wang, B., Zhao, X., Ponce, K., Qian, Q., & Ye, G. (2017). Association mapping of ferrous, zinc, and aluminum tolerance at the seedling stage in *indica* rice using MAGIC populations. *Frontiers in Plant Science*, *8*, 1822.

Miyadate, H., Adachi, S., Hiraizumi, A., Tezuka, K., Nakazawa, N., Kawamoto, T., ... Satoh-Nagasawa, N. (2011). OsHMA3, a P_{1B}-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. *New Phytologist*, *189*(1), 190-199.

Moore, K. L., Chen, Y., van de Meene, A. M. L., Hughes, L., Liu, W., Geraki, T., … Zhao, F. J. (2014). Combined NanoSIMS and synchrotron X-ray fluorescence reveal distinct cellular and subcellular distribution patterns of trace elements in rice tissues. *New Phytologist*, *201*(1), 104-115.

Mustafa, G., Ehsanullah, E., Akbar, N., Qaisrani, S., Iqbal, A., Khan, H., … Atta, B. (2011). Effect of zinc application on growth and yield of rice (*Oryza sativa* L.). *International Journal for Agro Veterinary and Medical Sciences*, *5*(6), 530-535.

Naik, S. K., & Das, D. K. (2007). Effect of split application of zinc on yield of rice (*Oryza sativa* L.) in an inceptisol. *Archives of Agronomy and Soil Science*, *53*(3), 305-313.

Nayak, S. C., Sahu, S. K., Rout, D. P., & Nayak, R. K. (2008). Suitable rice varieties for iron toxic soils of Orissa. *ORYZA*, *45*(2), 163-165.

Nestler, J., & Wissuwa, M. (2016). Superior root hair formation confers root efficiency in some, but not all, rice genotypes upon P deficiency. *Frontiers in Plant Science*, *7*, 1935.

Nestler, J., Keyes, S. D., & Wissuwa, M. (2016). Root hair formation in rice (*Oryza sativa* L.) differs between root types and is altered in artificial growth conditions. *Journal of Experimental Botany*, *67*(12), 3699-3708.

Nguyen, B. D., Brar, D. S., Bui, B. C., Nguyen, T. V., Pham, L. N., & Nguyen, H. T. (2003). Identification and mapping of the QTL for aluminum tolerance introgressed from the new source, *Oryza rufipogon* Griff., into *indica* rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *106*(4), 583-593.

Nguyen, V. T., Burow, M. D., Nguyen, H. T., Le, B. T., Le, T. D., & Paterson, A. H. (2001). Molecular mapping of genes conferring aluminum tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *102*(6-7), 1002-1010.

Nguyen, V., Nguyen, B., Sarkarung, S., Martinez, C., Paterson, A., & Nguyen, H. (2002). Mapping of genes controlling aluminum tolerance in rice: Comparison of different genetic backgrounds. *Molecular Genetics and Genomics*, *267*(6), 772-780.

Ni, J. J., Wu, P., Senadhira, D., & Huang, N. (1998). Mapping QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *97*(8), 1361-1369.

Norton, G. J., Deacon, C. M., Xiong, L., Huang, S., Meharg, A. A., & Price, A. H. (2010). Genetic mapping of the rice ionome in leaves and grain: Identification of QTLs for 17 elements including arsenic, cadmium, iron and selenium. *Plant and Soil*, *329*(1-2), 139-153.

Norton, G. J., Douglas, A., Lahner, B., Yakubova, E., Guerinot, M. Lou, Pinson, S. R. M., … Price, A. H. (2014). Genome wide association mapping of grain arsenic copper molybdenum and zinc in rice (*Oryza sativa* L.) grown at four international field sites. *PLOS ONE*, *9*(2), e89685.

Nozoe, T., Agbisit, R., Fukuta, Y., Rodriguez, R., & Yanagihara, S. (2008). Characteristics of iron tolerant rice lines developed at IRRI under field conditions. *Japan Agricultural Research Quarterly*, *42*(3), 187-192.

Ouyang, Y., Zeng, F., Zhuang, J., Yu, S., Zhu, L., Jin, Q., & Zhang, G. (2007). Genetic analysis of genotype × iron nutrition interaction on coleoptile elongation rate in rice (*Oryza sativa* L.). *Euphytica*, *156*(3), 311-318.

Ozanne, P. (1955). The effect of nitrogen on zinc deficiency in subterranean clover. *Australian Journal of Biological Sciences*, *8*(1), 47-55.

Phattarakul, N., Rerkasem, B., Li, L. J., Wu, L. H., Zou, C. Q., Ram, H., … Cakmak, I. (2012). Biofortification of rice grain with zinc through zinc fertilization in different countries. *Plant and Soil*, *361*(1-2), 131-141.

Ponnamperuma, F. N. (1972). The chemistry of submerged soils. *Advances in Agronomy*, *24*(C), 29-96.

Ponnamperuma, F. N., Bradfield, R., & Peech, M. (1955). Physiological disease of rice attributable to iron toxicity. *Nature*, *175*(4449), 265.

Prasad, R., Shivay, Y., & Kumar, D. (2016). Interactions of zinc with other nutrients in soils and plants - A review. *Indian Journal of Fertilisers*, *12*(5), 16-26.

Quijano-Guerta, C., Kirk, G. J. D., Portugal, A. M., Bartolome, V. I., & McLaren, G. C. (2002). Tolerance of rice germplasm to zinc deficiency. *Field Crops Research*, *76*(2-3), 123-130.

Ram, H., Sohu, V., Cakmak, I., & Singh, K. (2015). Agronomic fortification of rice and wheat grains with zinc for nutritional security. *Current Science*, *109*(6), 1171-1176.

Rana, W., & Kashif, S. (2014). Effect of different zinc sources and methods of application on rice yield and nutrients concentration in rice grain and straw. *Journal of Environmental and Agricultural Sciences*, *1*(9), 1-5.

Raven, J. A., & Taylor, R. (2003). Macroalgal growth in nutrient-enriched estuaries: A biogeochemical and evolutionary perspective. *Water, Air and Soil Pollution: Focus*, *3*(1), 7-26.

Rehman, H., Aziz, T., Farooq, M., Wakeel, A., & Rengel, Z. (2012). Zinc nutrition in rice production systems: A review. *Plant and Soil*, *361*(1-2), 203-226.

Rengel, Z., Batten, G., & Crowley, D. (1999). Agronomic approaches for improving the micronutrient density in edible portions of field crops. *Field Crops Research*, *60*, 27-40.

Ricachenevsky, F. K., Sperotto, R. A., Menguer, P. K., & Fett, J. P. (2010). Identification of Fe-excess-induced genes in rice shoots reveals a WRKY transcription factor responsive to Fe, drought and senescence. *Molecular Biology Reports*, *37*(8), 3735-3745.

Richardson, A. E., Hocking, P. J., Simpson, R. J., & George, T. S. (2009). Plant mechanisms to optimize access to soil phosphorus. *Crop and Pasture Science*, *60*(2), 124.

Rose, T. J., Impa, S. M., Rose, M. T., Pariasca-Tanaka, J., Mori, A., Heuer, S., … Wissuwa, M. (2013). Enhancing phosphorus and zinc acquisition efficiency in rice: A critical review of root traits and their potential utility in rice breeding. *Annals of Botany*, *112*(2), 331-345.

Rout, G. R., & Das, P. (2003). Effect of metal toxicity on plant growth and metabolism: I. Zinc. *Agronomie*, *23*(1), 3-11.

Ruengphayak, S., Ruanjaichon, V., Saensuk, C., Phromphan, S., Tragoonrung, S., Kongkachuichai, R., & Vanavichit, A. (2015). Forward screening for seedling tolerance to Fe toxicity reveals a polymorphic mutation in ferric chelate reductase in rice. *Rice*, *8*(1), 3.

Sadeghzadeh, B. (2013). A review of zinc nutrition and plant breeding. *Journal of Soil Science and Plant Nutrition*, *13*(4), 905-927.

Sahrawat, K. L. (2004). Iron toxicity in wetland rice and the role of other nutrients. *Journal of Plant Nutrition*, *27*(8), 1471-1504.

Sahrawat, K. L. (2008). Soil fertility advantages of submerged rice cropping systems. *Journal of Sustainable Agriculture*, *31*(3), 5-23.

Sahrawat, K. L. (2010). Reducing iron toxicity in lowland rice with tolerant genotypes and plant nutrition. *Plant Stress*, *4*(2), 70-75.

Sahrawat, K. L., Mulbah, C. K., Diatta, S., Delaune, R. D., Patrick, W. H., Singh, B. N., & Jones, M. P. (1996). The role of tolerant genotypes and plant nutrients in the management of iron toxicity in lowland rice. *The Journal of Agricultural Science*, *126*(02), 143.

Sahrawat, K., & Sika, M. (2002). Comparative tolerance of *Oryza sativa* and *O. glaberrima* rice cultivars for iron toxicity in West Africa. *International Rice Research Notes*, *27*(2), 30-31.

Septiningsih, E. M., Pamplona, A. M., Sanchez, D. L., Neeraja, C. N., Vergara, G. V, Heuer, S., … Mackill, D. J. (2009). Development of submergence-tolerant rice cultivars: The *Sub1* locus and beyond. *Annals of Botany*, *103*(2), 151-160.

Shi, J., Li, L., & Pan, G. (2009). Variation of grain Cd and Zn concentrations of 110 hybrid rice cultivars grown in a low-Cd paddy soil. *Journal of Environmental Sciences*, *21*(2), 168-172.

Shi, R., Zhang, Y., Chen, X., Sun, Q., Zhang, F., Römheld, V., & Zou, C. (2010). Influence of long-term nitrogen fertilization on micronutrient density in grain of winter wheat (*Triticum aestivum* L.). *Journal of Cereal Science*, *51*(1), 165-170.

Shimizu, A., Guerta, C., Gregorio, G., & Ikehashi, H. (2005). Improved mass screening of tolerance to iron toxicity in rice by lowering temperature of culture solution. *Journal of Plant Nutrition*, *28*(9), 1481-1493.

Shimizu, A., Kato, K., Komatsu, A., Motomura, K., & Ikehashi, H. (2008). Genetic analysis of root elongation induced by phosphorus deficiency in rice (*Oryza sativa* L.): Fine QTL mapping and multivariate analysis of related traits. *Theoretical and Applied Genetics*, *117*(6), 987-996.

Shimizu, A., Yanagihara, S., Kawasaki, S., & Ikehashi, H. (2004). Phosphorus deficiency-induced root elongation and its QTL in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *109*(7), 1361-1368.

Simoes, C. C., Melo, J. O., Magalhaes, J. V., & Guimaraes, C. T. (2012). Genetic and molecular mechanisms of aluminum tolerance in plants. *Genetics and Molecular Research Journal*, *11*(3), 1949-1957.

Singh, B., Natesan, S. K. A., Singh, B. K., & Usha, K. (2005). Improving zinc efficiency of cereals under zinc deficiency. *Current Science*, *88*(1), 36-44

Stein, R. J., Ricachenevsky, F. K., & Fett, J. P. (2009). Differential regulation of the two rice ferritin genes (*OsFER1* and *OsFER2*). *Plant Science*, *177*(6), 563-569.

Swamy, B. P. M., Rahman, M. A., Inabangan-Asilo, M. A., Amparado, A., Manito, C., Chadha-Mohanty, P., … Slamet-Loedin, I. H. (2016). Advances in breeding for high grain zinc in rice. *Rice*, *9*(1), 49.

Tanaka, A., Loe, R., & Navasero, S. A. (1966). Some mechanisms involved in the development of iron toxicity symptoms in the rice plant. *Soil Science and Plant Nutrition*, *12*(4), 32-38.

Vigueira, C. C., Small, L. L., & Olsen, K. M. (2016). Long-term balancing selection at the phosphorus starvation tolerance 1 (*PSTOL1*) locus in wild, domesticated and weedy rice (*Oryza*). *BMC Plant Biology*, *16*(1), 101.

Wan, J. L., Zhai, H. Q., & Wan, J. M. (2005). Mapping of QTLs for ferrous iron toxicity tolerance in rice (*Oryza sativa* L.). *Acta Genetica Sinica*, *32*(11), 1156-1166.

Wan, J., Zhai, H., Wan, J., & Ikehashi, H. (2003). Detection and analysis of QTLs for ferrous iron toxicity tolerance in rice, *Oryza sativa* L. *Euphytica*, *131*(2), 201-206.

Welch, R. M., & Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany*, *55*(396), 353-364.

Welch, R. M., & Norvell, W. A. (1993). Growth and nutrient uptake by barley (*Hordeum vulgare* L. cv Herta): Studies using an N-(2-Hydroxyethyl) ethylenedinitrilotriacetic acid-buffered nutrient solution technique (I. Zinc ion requirement). *Plant Physiology*, *101*(2), 619-625.

Wissuwa, M., & Ae, N. (2001). Further characterization of two QTLs that increase phosphorus uptake of rice (*Oryza sativa* L.) under phosphorus deficiency. *Plant and Soil*, *237*(2), 275-286.

Wissuwa, M., Ismail, A. M., & Graham, R. D. (2008). Rice grain zinc concentrations as affected by genotype, native soil-zinc availability, and zinc fertilization. *Plant and Soil*, *306*(1–2), 37-48.

Wissuwa, M., Ismail, A. M., & Yanagihara, S. (2006). Effects of zinc deficiency on rice growth and genetic factors contributing to tolerance. *Plant Physiology*, *142*(2), 731-741.

Wissuwa, M., Wegner, J., Ae, N., & Yano, M. (2002). Substitution mapping of *Pup1*: A major QTL increasing phosphorus uptake of rice from a phosphorus-deficient soil. *Theoretical and Applied Genetics*, *105*(6-7), 890-897.

Wissuwa, M., Yano, M., & Ae, N. (1998). Mapping of QTLs for phosphorus-deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, *97*(5-6), 777-783.

Wu, L. B., Shhadi, M. Y., Gregorio, G., Matthus, E., Becker, M., & Frei, M. (2014). Genetic and physiological analysis of tolerance to acute iron toxicity in rice. *Rice*, *7*(1), 8.

Wu, L. B., Ueda, Y., Lai, S. K., & Frei, M. (2017). Shoot tolerance mechanisms to iron toxicity in rice (*Oryza sativa* L.). *Plant, Cell and Environment*, *40*(4), 570-584.

Wu, P., Hu, B., Liao, C. Y., Zhu, J. M., Wu, Y. R., Senadhira, D., & Paterson, A. H. (1998). Characterization of tissue tolerance to iron by molecular markers in different lines of rice. *Plant and Soil*, *203*(2), 217-226.

Wu, P., Liao, C. Y., Hu, B., Yi, K. K., Jin, W. Z., Ni, J. J., & He, C. (2000). QTLs and epistasis for aluminum tolerance in rice (*Oryza sativa* L.) at different seedling stages. *Theoretical and Applied Genetics*, *100*(8), 1295-1303.

Xia, J., Yamaji, N., Che, J., Shen, R. F., & Ma, J. F. (2014). Differential expression of *Nrat1* is responsible for Al-tolerance QTL on chromosome 2 in rice. *Journal of Experimental Botany*, *65*(15), 4297-4304.

Xue, Y., Jiang, L., Su, N., Wang, J. K., Deng, P., Ma, J. F., … Wan, J. M. (2007). The genetic basic and fine-mapping of a stable quantitative-trait loci for aluminum tolerance in rice. *Planta*, *227*(1), 255-262.

Xue, Y., Wan, J., Jiang, L., Wang, C., Liu, L., Zhang, Y., & Zhai, H. (2006). Identification of quantitative trait loci associated with aluminum tolerance in rice (*Oryza Sativa* L.). *Euphytica*, *150*(1-2), 37-45.

Yamaji, N., Huang, C. F., Nagao, S., Yano, M., Sato, Y., Nagamura, Y., & Ma, J. F. (2009). A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. *The Plant Cell*, *21*(10), 3339-3349.

Yilmaz, A., Ekiz, H., Torun, B., Gultekin, I., Karanlik, S., Bagci, S. A., & Cakmak, I. (1997). Effect of different zinc application methods on grain yield and zinc concentration in wheat cultivars grown on zinc-deficient calcareous soils. *Journal of Plant Nutrition*, *20*(4-5), 461-471.

Zhai, Z., Gayomba, S. R., Jung, H.- ., Vimalakumari, N. K., Pineros, M., Craft, E., … Vatamaniuk, O. K. (2014). OPT_3 is a phloem-specific iron transporter that is essential for systemic iron signaling and redistribution of iron and cadmium in *Arabidopsis*. *The Plant Cell*, *26*(5), 2249-2264.

Zhang, J., Aijaz, A. S., Chai, L., Cui, Y., Wang, X., Zheng, T., … Li, Z. (2013). Mapping of QTL for iron and zinc toxicity tolerance at seedling stage using a set of reciprocal introgression lines of rice. *Acta Agronomica Sinica*, *39*(10), 1754-1765.

Zhang, P., Zhong, K., Tong, H., Shahid, M. Q., & Li, J. (2016). Association mapping for aluminum tolerance in a core collection of rice landraces. *Frontiers in Plant Science*, *7*, 1415.

Zhang, X., Zhang, G., Guo, L., Wang, H., Zeng, D., Dong, G., … Xue, D. (2011). Identification of quantitative trait loci for Cd and Zn concentrations of brown rice grown in Cd-polluted soils. *Euphytica*, *180*(2), 173-179.

Zhou, X., & Yang, Y. (2004). Differential expression of rice *Nramp* genes in response to pathogen infection, defense signal molecules and metal ions. *Physiological and Molecular Plant Pathology*, *65*(5), 235-243.

Zygalakis, K. C., Kirk, G. J. D., Jones, D. L., Wissuwa, M., & Roose, T. (2011). A dual porosity model of nutrient uptake by root hairs. *New Phytologist*, *192*(3), 676-688.

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Evaluation of Glyphosate Levels in Sediments of Milky Stork Foraging Areas in Kuala Gula Bird Sanctuary, Perak, Malaysia

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ABSTRACT

Glyphosate is the most widely used herbicide in the world since its first introduction in the 1970s. Although its accumulation and direct impact on top predators are unlikely, it has the potential to affect the lower trophic community causing imbalance to the local food web. This paper highlights the background levels of glyphosate in the sediment in a part of the Important Bird and Biodiversity Programme Areas (IBA) in the Matang coast i.e., the Kuala Gula Bird Sanctuary, Perak, Malaysia. Despite housing endangered species like the milky stork and migratory birds, the area is surrounded by massive oil palm plantations that utilize glyphosate-based herbicide. Sediment samples were taken from areas frequently visited by the bird population. The herbicide was analyzed by HPLC-UV detection with previous derivatization using 9-fluorenylmethylchloroformate (FMOC-Cl). In general, the glyphosate levels were between 0.26 and 1.72 ppm. These levels are considered low when compared to other agricultural sites in both local and regional areas. In addition, no significant relationship was established between glyphosate levels in the study sites with the distance from nearby agricultural activity ($r = 0.2$, $n = 18$, $p > 0.05$). This evidence suggests that the pollutant most likely comes from an inter-connected network of water canals and water-bodies that accumulate the plantations' effluent over time. Due to its resistant nature and low concentration in the field, there is

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little concern over its adverse impact on the aquatic ecosystem in the study area. However, continuous monitoring is still emphasized in this paper.

Keywords: Agriculture, glyphosate, Kuala Gula Bird Sanctuary, Malaysia, milky stork

INTRODUCTION

Glyphosate is the most widely used herbicide in the world since its first introduction in the 1970s. Oil palm plantation is one of the most important industries that utilize this herbicide. At least 15 million liters of this herbicide is used annually to control the weed problem (Raman et al., 2010). Although its accumulation and direct impact on top predators are unlikely, it has the potential to affect the lower trophic community causing imbalance to the local food web. In addition, there is growing evidence that glyphosate and its metabolite are harmful. Its widespread use has caused negative effects to the environment (Székács & Darvas, 2012). Although glyphosate itself is only slightly or moderately toxic to the aquatic animals (Giesy et al., 2000; Solomon & Thompson, 2003), the surfactant polyoxyethylene amine (POEA) added in the commercial formulation is considered to be more toxic (Brausch & Smith, 2007; Tsui & Chu, 2003). Thus the combination of the two is expected to be more lethal (Yusof et al., 2014).

Williams et al. (2012) stated that glyphosate was non-volatile and bound strongly to most soils or sediments, and was degraded by microbes before it could reach any non-target area. The C-P bonds in the phosphonate of this herbicide are resistant to physicochemical factors (Murai & Tomizawa 1976) but they can be cleaved by the microbial enzymatic system under certain conditions (Kononova & Nesmeyanova, 2002). In addition, the degradation of glyphosate is slow in muck soils due to the inaccessibility of any microbial metabolism into the muck (Cheah et al., 1998). Similarly, sediment could represent an alternative transport pathway during monsoonal floods which move vast quantities of sediment with glyphosate in them (Brodie et al., 2012; Kroon et al., 2012).

Malaysia is the second largest oil palm producer behind Indonesia (Malaysian Oil Palm Council [MPOC], 2016) and the industry relies heavily on herbicides to control weeds. Mishandling and excess usage of glyphosate-based herbicides in the country have also been reported (Ali & Shaari, 2015). However, monitoring data pertaining to glyphosate levels in the environment is scarce in Malaysia. Limited study has been conducted so far to address this issue. For instance, high concentration of glyphosate in waterbodies was reported in Tasik Chini particularly during the rainy season (Mardiana-Jansar & Ismail, 2014). However, no other monitoring studies were conducted to highlight this issue. Glyphosate can be easily leached out into the aquatic environment following high rainfall activity. Rainfall in Malaysia is divided into two maximum rainfalls i.e., between October and November as well as April and May for the most part of the peninsular with an average of 250 cm of rain annually (Malaysian Meteorological Department [MetMalaysia], 2017). Thus, more studies are needed to monitor and understand the impact of the herbicide's input into such an environment. In addition, with the heavy usage of glyphosate-based herbicides in the country, leaching and surface runoff will occur during heavy rainfall activity causing most of it to flow into rivers and finally enter the sea. Interestingly, glyphosate has also been demonstrated to be moderate to highly persistent in marine water thus affecting coral reefs in the ocean (Mercurio et al., 2014).

The Kuala Gula Bird Sanctuary in Malaysia is an important example where intense agricultural activity in its hinterland could potentially cause a negative impact on its aquatic environment. Given the large scale plantations operating with over 7000 ha that reach Kuala Sepetang in the east and Bagan Serai on the north, as well as their proximity to the Kuala Gula's estuary and mudflats, the accumulation of glyphosate in the sediment can potentially be harmful to lower trophic organisms. These include benthic and fish communities that are mostly consumed by top predators like the water birds in the area. Consequently, the decline of their prey would greatly affect the predator population in the long run. However, there are no available studies that highlight this particular issue in this area. Considering the importance of the sanctuary to both resident and migratory birds, including endangered species like the milky stork, there is a need to evaluate the levels of glyphosate in Kuala Gula's aquatic environment. The findings would also serve as important baseline information for future studies.

MATERIALS AND METHODS

Study Area

The Kuala Gula Bird Sanctuary (4°56'00" N; 100°28'00" E) is located in the northern part of the west coast of Peninsular Malaysia in the Kerian district, 45 kilometres from Taiping, Perak (Figure 1). It is also part of the north area of the Matang Mangrove Forest Reserve (MMFR), the single largest track of mangroves in the country with an area of approximately 40,000 ha. Elevation of the area averages at 2 - 3 metres above sea level and the average rainfall is 2063 mm per annum (Department of Irrigation and Drainage Malaysia [JPS], 2018). Kuala Gula is regarded as one of the important stopovers in the East Asia-Australia flyway and also a sanctuary to a number of endangered species such as the milky stork and lesser adjutant (Ismail & Rahman, 2016). Possessing one of the major estuaries in the Matang coast, it is included in the Important Bird and Biodiversity Area (IBA) Programme, a worldwide initiative aimed at identifying, documenting and working towards the conservation and sustainable management of a network of critical sites for the world's birds. Continuous habitat destruction along the coastal area of the peninsular is considered as one of the primary factors that drive the milky storks in Malaysia close to extinction (Ismail & Rahman, 2012, 2016). As such, Kuala Gula serves as an important and final frontier in which the last population could thrive. Therefore, monitoring its environment to ensure any development and human activity are being carried out sustainably should remain a top priority. At least five sites frequently visited by the endangered milky stork population were studied. They consisted of both natural and artificial terrains and hydrology which serve as important foraging areas for the milky storks (Rahman et al., 2017). In addition, some of these sites also overlap with the wintering ground of many shorebirds during the migratory season. Table 1 shows the descriptions of the sampling areas in Kuala Gula. Palm oil plantation is a massive industry in Kuala Gula where most of the inland areas have been converted to support this agricultural activity. It is estimated that at least 7,000 ha of palm oil plantations are active throughout Kuala Gula. The

Table 1
Description of the study sites in Kuala Gula Bird Sanctuary, Malaysia

Site	Coordinates	Site description
S1	4°56015.77N; 100°28003.39E	Water canal that channels both agricultural and domestic effluents into the Gula River’s tributary
S2	4°56024.22N; 100°28007.42E	Intertidal mudflat area close to a jetty and fishing village in Gula River
S3	4°57010.24N; 100°29013.66E	Intertidal mudflat turned into shrimp farm
S4	4°55040.03N; 100°27046.54E	Intertidal mudflat of Gula River that also receives effluents from nearby aquaculture farms
S5	4°56014.14N; 100°28006.68E	Intertidal mudflat adjacent to a vacation resort along the Gula River
S6	4°55’52.12”N; 100°29’24.71”E	Mangrove area recently turned into new shrimp farms in Pulau Gula (Gula Island)

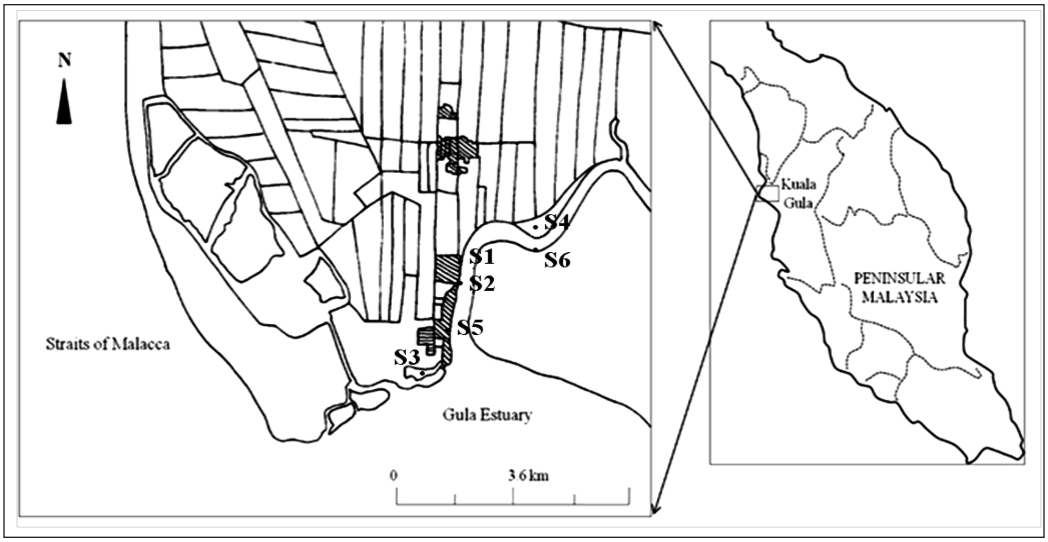


Figure 1. Map of the Kuala Gula Bird Sanctuary, Perak, Malaysia

aquaculture industry is also expanding rapidly and has led to an increased number of reclaimed mangroves in recent years (Ismail & Rahman, 2016; Rahman et al., 2013, 2017).

Laboratory Work and Analysis

Sampling activity was carried out in Kuala Gula's coastal in January 2013. Approximately 50 g of surface sediments (1-5 cm depth) were taken from each site, S1–S6 and kept in an ice box with a temperature of 4^{o}C during transportation. At the laboratory, the samples were dried in an air-circulating oven at 60^{o}C for at least 72 hours or until constant dry weights were achieved (Ismail & Ramli, 1997). The samples were later sieved for further processing (Fisher Scientific Company, mesh size: 63 μm). For the glyphosate extraction procedure, a modified method based on Peruzzo et al. (2008) was employed. The presence of the herbicide in the sediment was determined after the extraction of 15 g of sample with monopotassium phosphate (MKP, KH_2PO_4) 0.1 M; the mixture then undergoes agitation for 15 min followed by centrifugation (1,464 *g*; 10 min) and later filtration through Whatman No. 1 filter paper. The extraction was repeated twice on solid residue, obtaining a 25 ml extract from each sample. Later, the extracts were filtered through a 0.45 mm cellulose acetate membrane. Finally, the derivatization process was carried out as follows: 0.25 ml of Borate buffer 0.025 M, 0.30 ml of Fmoc-Cl 2 M and chloroform ($CHCl_3$) were added to 1 ml of each sample. Then the mixture was left for 24 hours to react in 40°C temperature in the dark. After that, 0.30 ml of Phosphoric acid (H_3PO_4) (2%) was added and then kept in the fridge for further analysis. The derivatized product (Gly-Fmoc) was analyzed in triplicates using High Performance Liquid Chromatography (HPLC, Shimadzu) with Ultra-Violet (UV) detector; 20 µL of acetonitrile was injected as blank solution prior to the samples, after which each sample was injected three times with a similar volume as the blank. The limit of detection for the glyphosate analyzed was 0.00021 nanogram per gram (ng/g), with retention time of 32.6 minutes. The chromatographic conditions used in this study were as follow: Fortis Universal C18 column (5 mm particle size, length I.D.: 15 cm x 4.9 mm); mobile phase 0.05 M phosphate buffer (pH 5.5): acetonitrile (70:30); flow: 0.8 ml/min; and UV detection: 206 nm. The standards used were derived using Sigma PESTANAL® with 99% purity and further diluted using Milli-Q® water. The glyphosate was first dissolved into 100 ppm concentration and later had its concentration diluted to 1 ppm, 0.5 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, 0.005 ppm and 0.001 ppm for testing. Figure 2 shows the peak versus the concentration of the glyphosate's standard analyzed for linearity purpose. Other chemicals used are of analytical grade quality. Recoveries of 80-120% were obtained for the glyphosate in the sediments. Pearson's rho correlation was used to understand the relationship between the glyphosate levels and the distance from agricultural activity in the area.

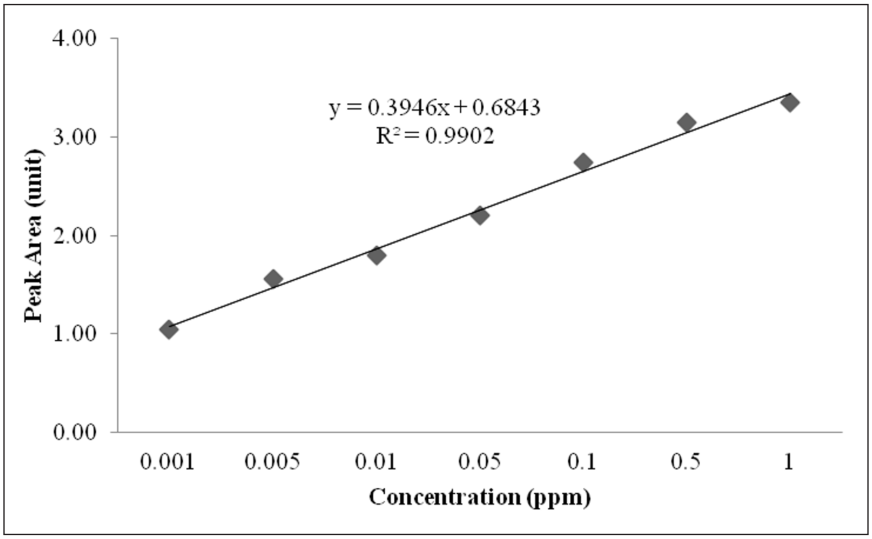
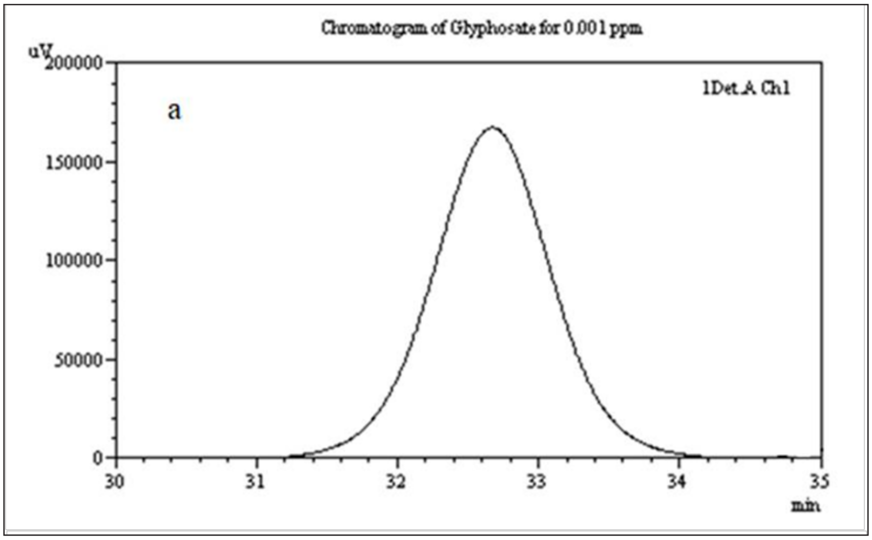


Figure 2. Peak area versus glyphosate's standards linearity

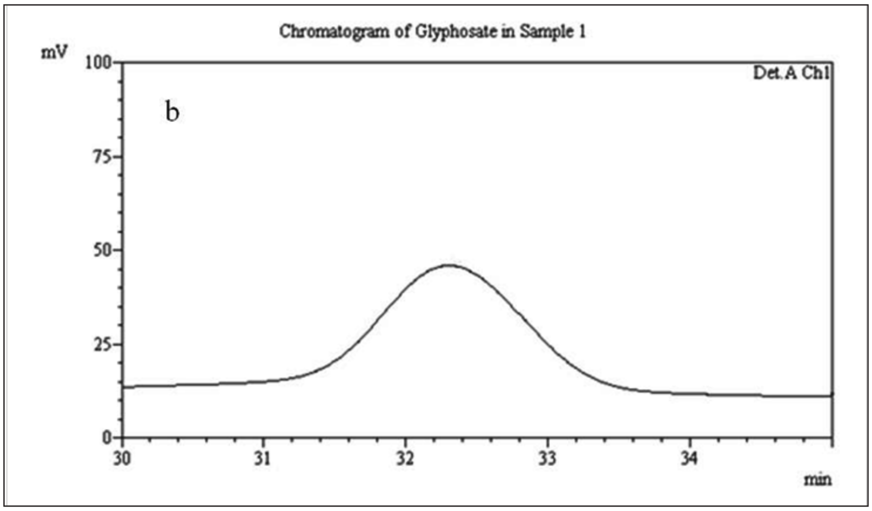
RESULTS AND DISCUSSION

The glyphosate was successfully extracted and analyzed from surface sediments in the foraging areas of Kuala Gula. Figure 3 shows the example of the chromatograms for the standard (0.001 ppm) and one of the samples analyzed. In general, the levels obtained ranged from 0.26 to 1.80 ppm. Figure 4 shows the different levels of glyphosate according to the different sites available. In general, sites 1 to 4 had accumulated higher amounts of glyphosate in the sediment compared to sites 5 and 6. These sites (S1, S2, S3 and S4) are mostly surrounded by massive oil palm plantations and could have directly or indirectly received effluents from the agricultural activity. On the other hand, S6 had the lowest glyphosate level with 0.26 ppm recorded in the sediment. We found no significant correlation between the glyphosate levels and the distance to the oil palm plantations in the area ($r = 0.2$, $n = 18$, $p > 0.05$). The findings suggest that the pollutant most likely comes from an inter-connected network of water canals and water-bodies surrounding the study sites which accumulate effluents from agricultural activity in Kuala Gula. This could occur either through a leaching process or direct input of the effluent particularly during high rainfall activity. Further study could focus on monitoring the temporal fluctuation of glyphosate levels in different seasons post-application in the field to understand its accumulation and distribution patterns.

According to Smith and Neiman (2012), soils are vertically weathering profiles that develop in place requiring time and stable surface while sediments are particles transported by natural forces like wind or water. Schuette (1998) stated that glyphosate was relatively immobile in most soil environments as a result of its strong adsorption to soil particles. In addition, glyphosate can also last for at least one year following aerial-spray



(a)



(b)

Figure 3. Example of the chromatogram for a) 0.001 ppm standard and b) one of the samples in which glyphosate compound was detected at the 32.6 minutes mark

activity (Major et al., 2003) depending on the soil type and light availability. On the contrary, glyphosate in sediment, although can be very resistant, is highly mobile as it can even be found in areas that have no direct pesticide application which are considered undeveloped and remote (Smalling et al., 2012). Its strong binding particularly to organic matter allows for long distance transport and being persistent in the environment (Solomon & Thomspon, 2003). Moreover, as sediment appears to be the major sink for glyphosate residue in most of the aquatic systems (Schuette, 1998), this pollutant can reach farther into seawater, even affecting coral reefs such as

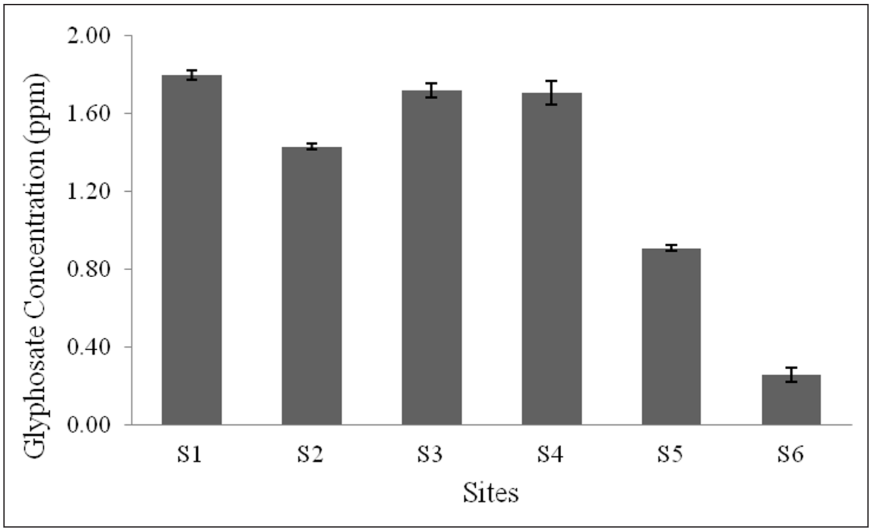


Figure 4. Glyphosate concentrations (mean ± standard deviation) in sediment in the different sites used by the Milky stork in Kuala Gula

in the case of the Great Barrier Reef studied by Mercurio et al. (2014). In our study, the presence of this agricultural pollutant can be explained by the scale of the oil palm plantations in Kuala Gula, estimated to accommodate more than 7000 ha area. Moreover, given that Malaysia is the second largest oil palm producer after Indonesia, which accounts for 39% of global oil palm production and 44% of global export (MPOC, 2016), the use of such herbicide is heavily relied upon. For instance, a survey conducted by Abdul (2017) estimated that the total amount of glyphosate-based herbicide volume sold in the country in 2017 alone was about 27 kilotons. The rampant mismanagement of the glyphosate-based herbicide in the country involving unlicensed manufacturers and retailers, and inadequate users' information that lead to the mishandling and excess usage of the pollutant further contribute to the problem (Ali & Shaari, 2015). As rainfall activity in Malaysia is often very high, much of this herbicide will be washed out into the streams and rivers. Thus, the possibility of the pollutant reaching remote areas needs to be studied to provide important information on the pollutant's mobility in the larger part of the Matang Mangrove Forest.

Table 2 shows the levels of glyphosate in sediments in agricultural areas from different countries worldwide using comparable methodology. In general, glyphosate levels recorded in Kuala Gula is much lower compared to other areas with agricultural activity of similar intensity. Soil characteristics may play an important role in the accumulation of glyphosate as glyphosate degradation rates vary considerably across a wide variety of soil types but it does not appear to be largely dependent on soil pH or organic content (Grossbard & Atkinson, 1984). Sprankle et al. (1975) reported that the prime factor in determining the amount of glyphosate adsorbed to soil particles was the soil phosphate level and that glyphosate was bound to soil through the phosphonic acid moiety. Phosphate (P) in mangrove soils can be immobile and unavailable for plant use (Reef et al., 2010) but the occurrence of phosphate-solubilizing bacteria in association with mangrove roots serve as an important role in P uptake by the plant (Kothamasi et al., 2006; Vazquez et al., 2000). Although the soil properties in the study areas were not studied, the milky stork population was often found foraging close to or along the mangroves which are muddy and have high organic content. The half-life of glyphosate in both water and soil can range from few days to up a year depending on the soil composition (Székács & Darvas, 2012). For instance, soil parameters and temperature differ significantly depending on its composition, making glyphosate half-lives reaching more than 300 and up to 428 days (Borggard & Gimsing, 2008). In addition, due to its adsorption on clay particles and organic matter, glyphosate may remain unchanged in the soil for varying lengths of time (Penaloza-Vazquez et al., 1995). The presence of high organic matter in the mangrove would mean that most of the available glyphosate residues will bind to them and are less likely to be leached into the aquatic environment. Hence the sediment samples obtained from such areas may explain the low level of glyphosate recorded in this study. There are also many tributaries or rivers nearby in which the glyphosate could escape into eventually reaching and sinking into the estuaries. This may help reduce the amount of glyphosate found in a particular area. As such, to account for the actual amount of glyphosate in the environment in the area, future studies should also consider obtaining samples from other nearby rivers and estuaries.

Water birds are known to be an important indicator for environmental changes and have been used widely around the world (Rahman & Ismail, 2018). Although the high levels of glyphosate may not affect the Milky stork population directly, its indirect effect on the lower trophic organisms including benthic and fish communities have been highlighted by many (Ayoola, 2008; Cuhra et al., 2013; Lajmanovich et al., 2003; Meyer et al., 2014; Paganelli et al., 2010; Relyea, 2005; Schneider et al., 2009). These studies showed that, through

Table 2
Comparison of glyphosate levels in sediments reported in different countries with commercial agricultural activity

No	Country	Agricultural activity	GLYP levels (ppm)	Reference(s)
1	**Kuala Gula, Malaysia**	**Palm oil**	**0.3 – 1.8**	**This study**
2	Tasik Chini, Malaysia	Palm oil	0.0 – 6.0	Mardiana-Jansar and Ismail (2014)
3	Pampa, Argentina	Soybean	0.5 – 5.0	Peruzzo et al. (2008)
4	Chaco, Argentina	Soybean/ Corn	0.0 – 0.3	Aparicio et al. (2014)
5	Willipa Bay, Washington, USA	NA	1.6 – 2.8	Paveglio et al. (1996)

various modes of actions, even low levels of glyphosate can negatively affected non-target organisms. In addition, Mertens et al. (2018) highlighted that the strong tendency of glyphosate to sorb on minerals through its functional groups might mobilize bound trace metals by chelation and sorbed anions by displacement. High glyphosate input in soils containing elevated concentrations of heavy metals and phosphate have been shown to increased leaching activity of the metals compared to the one with normal background concentrations of heavy metals (Barret & McBride, 2006). Through bio-accumulation and bio-magnification processes, the metals will affect the food chains and ultimately top predators like the water birds. As such, there is an important need to continuously monitor glyphosate activity in the study area. Currently, apart from the mismanagement of the herbicide by irresponsible parties, continuous activity and the expansion of anthropogenic activity in Kuala Gula could lead to the increase of glyphosate levels in the aquatic environment in the future. Such activities would have a negative impact on the water bird population such as the endangered milky storks as they rely on these sites as a food source. Thus, there is a need to increase the awareness and support among the public, land owners and related industries in the area to help conserve the coastal ecosystem of Kuala Gula. Nonetheless, at its current state, glyphosate pollution in Kuala Gula can still be considered as very low and is unlikely to cause any issues to the water birds in the near future.

CONCLUSION

The glyphosate levels in Kuala Gula are still considered safe and are unlikely to cause any negative impact on the endangered species in the area. However, further monitoring is still needed as the mismanagement of the herbicide and the reclamation of the mangrove and its surrounding areas still continue. The current findings also serve as important baseline information for future studies.

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REFERENCES

Abdul, N. R. (2017). Glyphosate position in Malaysia market. *Palma Journal*, *16*(1), 13-17.

Ali, A., & Shaari, N. (2015). Mismanagement of chemical agriculture in Malaysia from legal perspective. *Procedia Economics and Finance*, *31*, 640-650.

Aparicio, V. C., Gerónimo, E. D., Marino, D., Primost, J., Carriquiriborde, P., & Costa, J. L. (2014). Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. *Chemosphere*, *93*(9), 1866-1873.

Ayoola, S. O. (2008). Toxicity of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*) juvenile. *African Journal of Agricultural Research*, *3*(12), 825-834.

Barrett, K. A., & McBride, M. B. (2006). Trace element mobilization in soils by glyphosate. *Soil Science Society of America Journal*, *70*(6), 1882–1888.

Borggard, O. K., & Gimsing, A. L. (2008). Fate of glyphosate in soil and the possibility of leaching to ground and surface waters: A review. *Pest Management Science*, *64*(4), 441–456.

Brausch, J. M., & Smith, P. N. (2007). Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. *Archives of Environmental Contamination and Toxicology*, *52*(2), 217–221.

Brodie, J. E., Kroon, F. J., Schaffelke, B., Wolanski, E. C., Lewis, S. E., Devlin, M. J., … Davis, A. M. (2012). Terrestrial pollutant runoff to the Great Barrier Reef: An update of issues, priorities and management responses. *Marine Pollution Bulletin*, *65*(4-9), 81-100.

Cheah, U. B, Kirkwood, R. C., & Lum, K. Y. (1998). Degradation of four commonly used pesticides in Malaysian agricultural soils. *Journal of Agricultural and Food Chemistry*, *46*(3), 1217–1223.

Cuhra, M., Traavik, T., & Bøhn, T. (2013). Clone and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in *Daphnia magna*. *Ecotoxicology*, *22*(2), 251–262.

Department of Irrigation and Drainange Malaysia. (2018). *Rainfall manual: Ladang Gula, Kuala Kurau* (*Station no. 4904026*). Retrieved September 6, 2017, from http://h2o.water.gov.my

Giesy, J., Dobson, S., & Solomon, K. (2000). *Reviews of environmental contamination and toxicology: Ecotoxicological risk assessment for Roundup® herbicide*. New York, NY: Springer.

Grossbard, E., & Atkinson, D. (1984). *The herbicide glyphosate*. London, United Kingdom: Butterworth-Heinemann Ltd.

Ismail, A., & Rahman, F. (2016). Current status of the milky stork re-introduction in Malaysia and its challenges. *Tropical Life Sciences Research*, *27*(2), 13-24.

Ismail, A., & Ramli, R. (1997). Trace metals in sediments and mollusc from an estuary receiving pig effluents. *Environmental Technology*, *18*(1), 509-515.

Ismail, A., & Rahman, F. (2012). An urgent need for milky stork study in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, *35*(3), 407-412.

Kononova, S. V., & Nesmeyanova, M. A. (2002). Phosphonates and their degradation by microorganisms. *Bio-Chemistry*, *67*(2), 184-195.

Kothamasi D., Kothamasi S., Bhattacharyya, A., Kuhad, R., & Babu, C. (2006). Arbuscular mycorrhizae and phosphate solubilising bacteria of the rhizosphere of the mangrove ecosystem of Great Nicobar island, India. *Biology and Fertility of Soils*, *42*(4), 358-361.

Kroon, F. J., Kuhnert, P. M., Henderson, B. L., Wilkinson, S. N., Kinsey-Henderson, A., Abbott, B., & Turner, R. D. R. (2012). River loads of suspended solids, nitrogen, phosphorus and herbicides delivered to the Great Barrier Reef lagoon. *Marine Pollution Bulletin*, *65*(4-9), 167-181.

Lajmanovich, R. C., Sandoval, M. T., & Peltzer, P. M. (2003). Induction of mortality and malformation in *Scinax nasicus* tadpoles exposed to glyphosate formulations. *Bulletin of Environmental Contamination and Toxicology*, *70*(3), 612–618.

Major, W. W. III, Grue, C. E., Gardner, S. C., & Grassley, J. M. (2003). Concentrations of glyphosate and AMPA in sediment following operational applications of Rodeo® to control smooth cordgrass in Willapa Bay, Washington, USA. *Bulletin of Environmental Contamination and Toxicology*, *71*(5), 912-918.

Malaysian Meteorological Department. (2017). *General climate of Malaysia*. Retrieved January 6, 2017, from http://www.met.gov.my

Malaysian Oil Palm Council. (2016). *Malaysia palm oil industry*. Retrieved January 6, 2017, from www.mpoc.org.my

Mardiana-Jansar, K., & Ismail, B. S. (2014). Residue determination and levels of glyphosate in surface waters, sediments and soils associated with oil palm plantation in Tasik Chini, Pahang, Malaysia. *AIP Conference Proceedings*, *1614*(1), 795-802.

Mercurio, P., Flores, F., Mueller, J. F., Carter, S., & Negri, A. P. (2014). Glyphosate persistence in seawater. *Marine Pollution Bulletin*, *85*(2), 385-390.

Mertens, M., Höss, S., Neumann, G., Afzal, J., & Reichenbecher, W. (2018). Glyphosate, a chelating agent—Relevant for ecological risk assessment? *Environmental Science and Pollution Research International*, *25*(6), 5298-5317.

Meyer, E. C., Gress, S., Séralini, G.-É., & Denis, I. G. (2014). An acute exposure to glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear quality. *Environmental Toxicology and Pharmacology*, *38*(1), 131-140.

Murai, C., & Tomizawa, C. (1976). Chemical transformation of S-Benzyl *O*-Ethyl phenylphosphonothiolate (Inezin®) by ultraviolet light. *Journal of Environmental Science and Health*, *11*(2), 185–197.

Paganelli, A., Gnazzo, V., Acosta, H., Lopez, S. L., & Carrasco, A. E. (2010). Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signalling. *Chemical Research in Toxicology*, *23*(10), 1586-1595.

Paveglio, F. L., Kilbride, K. M., Crue, C. E., Simenstad, C. A., & Fresh, K. L. (1996). Use of Rodeo® and X-77® spreader to control smooth cordgrass (*Spartina alteniflora*) in a southwestern Washington estuary: 1. Environmental fate. *Environmental Toxicology and Chemistry*, *15*(6), 961–968.

Penaloza-Vazquez, A., Mena, G. L., Herrera-Estrella, L. & Bailey, A. M. (1995). Cloning and sequencing of the genes involved in glyphosate utilization by *Pseudomonas pseudomallei*. *Applied and Environmental Microbiology*, *61*(2), 538-543.

Peruzzo, P. J., Porta, A. A., & Ronco A. E. (2008). Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution*, *156*(1), 61-66.

Rahman, F., & Ismail, A. (2018). Waterbirds: An important bio-indicator of ecosystem. *Pertanika Journal of Scholarly Research Reviews*, *4*(1), 81-90.

Rahman, F., Ismail, A., & Yusof, S. (2013). Metals contamination in the foraging area of milky stork: Evidence of anthropogenic inputs in the aquatic environment of Kuala Gula, Malaysia. *Toxicological and Environmental Chemistry*, *95*(9), 1499-1505.

Rahman, F., Ismail, A., Omar, H., & Hussin, M. Z. (2017). Exposure of the endangered milky stork population to cadmium and lead via food and water intake in Kuala Gula Bird Sanctuary. *Toxicology Reports*, *4*(1), 502-506.

Raman, I. A. B, Shah, A. R. M. B., Sim, K. C., Ismail, R., Hassan, H. A., Shamsudin, B., & Tan, A. S. H. (2010). *Performance of palm oil adjuvants in glyphosate herbicide formulations for weed control*. Retrieved February 12, 2017, from http://palmoilis.mpob.gov.my/publications/TOT/TT-467&468.pdf

Reef, R., Feller, I. C., & Lovelock, C. E. (2010). Nutrition of mangroves. *Tree Physiology*, *30*(9), 1148-1160.

Relyea, R. A. (2005). The lethal impact of roundup on aquatic and terrestrial amphibians. *Ecological Applications*, *15*(4), 1118–1124.

Schneider, M. I., Sanchez, N., Pineda, S., Chi, H., & Ronco, A. (2009). Impact of glyphosate on the development, fertility and demography of

Chrysoperla externa (Neuroptera: Chrysopidae): Ecological approach. *Chemosphere*, *76*(10), 1451–1455.

Schuette, J. (1998). *Environmental fate of glyphosate: Environmental monitoring and pest management*. Retrieved September 12, 2017, from https://www.cdpr.ca.gov/docs/emon/pubs/fatememo/glyphos.pdf

Smalling, K. L., Orlando, J. L., Calhoun, D., Battaglin, W. A., & Kuivila, K. M. (2012). *Occurrence of pesticides in water and sediment collected from amphibian habitats located throughout the United States, 2009-2010*. Retrieved September 12, 2017, from https://pubs.usgs.gov/ds/707/pdf/ds707.pdf

Smith, K., & Neiman, F. (2012). *Soil or sediment? Horizons or deposits? Geoarchaeology to the rescue!* Retrieved January 7, 2017, from https://www.monticello.org/site/blog-and-community/posts/soil-or-sediment-horizons-or-deposits-geoarchaeology-rescue

Solomon, K. R., & Thompson, D. G. (2003). Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. *Journal of Toxicology and Environmental Health*, *Part B*, *6*(3), 289-324.

Sprankle, W., Meggitt W., & Penner. D. (1975). Rapid inactivation of glyphosate in the soil. *Weed Science*, *23*(3), 224-228.

Székács, A., & Darvas, B. (2012). Forty years with glyphosate herbicides. In A. G. H. Mohammed-Naguib (Ed.), *Herbicides: Properties, synthesis and control of weeds* (pp. 247-284). Rijeka, Croatia: IntechOpen.

Tsui, M. T. K., & Chu, L. M. (2003). Aquatic toxicity of glyphosate-based formulations: Comparison between different organisms and the effects of environmental factors. *Chemosphere*, *52*(7), 1189-1197.

Vazquez, P., Holguin, G., Puente, M. E., Lopez-Cortes, A., & Bashan, Y. (2000). Phosphate-solubilising microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biology and Fertility of Soils*, *30*(5-6), 460-468.

Williams, A. L., Watson, R. E., & De Sesso, J. M. (2012). Developmental and reproductive outcomes in humans and animals after glyphosate exposure: A critical analysis. *Journal of Toxicology and Environmental Health*, *15*(1), 39-96.

Yusof, S., Ismail, A., & Alias, M. S. (2014). Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Marine Pollution Bulletin*, *85*(2), 494-498.

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Presence of Heavy Metals in Feathers of Avian Species and in Soils of Barangay Ipil-Calancan Bay, Sta.Cruz, Marinduque Island, Philippines

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ABSTRACT

Heavy metals may contaminate food, water, and air and bring toxic effects to both wildlife and humans. This study sought to assess the concentrations of heavy metals in the feathers of avian species and in soils using X-ray fluorescence in Barangay Ipil-Calancan Bay, Sta. Cruz, Marinduque Island, Philippines. Iron, Cu, Zn, Mn, Cr, Sc, Pb, Sr, and Se were observed in all samples while Co and As were only found in sampled avian species. Among these heavy metals, the concentrations of As, Cr, Pb, Se, and Zn were beyond the critical levels which were reported in other studies. Highly significant concentrations of Cr, Cu, Fe, Sc, Sr, Zn, and Mn were observed in Philippine pied fantail, white browed crake, white-collared kingfisher and little heron. Arsenic accumulation was only observed in yellow-vented bulbul while Cu, Fe, Sr, Zn, and Cr were significantly lowest in pink-necked green pigeon and Tabon scrubfowl. The kingfisher, crake, scrubfowl, and fantail may be the important bioindicators of the area. This study found correlation between soil and feather heavy metal contaminations concerning Mo with Fe, Mn, Cr, V, Ti, and Sb, Mn with Rb and Sb, Se with Sc, and Zn with Sc. Heavy metals may cause security problems in the area such as with food,

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humans and ecosystems' health. The study also provides baseline data using a non-invasive technique for biomonitoring heavy metal contamination.

Keywords: Avian species, bioaccumulation, feathers, heavy metals, soils

INTRODUCTION

Heavy metals from mining activities are considered among the huge environmental problems as it may contaminate the soil, air, water, and food resources. Its bioaccumulation eventually harms wildlife and humans. Mining and ore processing ranked second among the top ten worst pollution industries in 2016 world's worst pollution problems reported by the Pure Earth and Green Cross Switzerland (2016). In humans, four of the top six toxic threats known were from heavy metals such as lead, mercury, chromium, and cadmium. These were estimated to put 66 million people at risk and accounted for 13,750,000 of Disability Life Years (DALYs) lost due to incapacitating, dreaded illnesses and mortality in developing nations (Pure Earth and Green Cross Switzerland, 2015). Accordingly, wildlife exposed to the heavy metal pollution were stunted, sensitive to parasites and pathogens, and of low survival and reproductive capacity. Considering these unwanted effects, it is important to study the present concentrations of heavy metals in areas affected by mining activities. This also helps monitor trends and evaluate ecological risks as well as provide information which is foremost in the decision making and provision of mitigating measures.

Bioaccumulation studies have been widely used along with other methods to determine the presence of heavy metals in the environment especially in the higher trophic levels. Bioaccumulation studies require a few samples and some tissues of organisms from the same area to provide precise information on the degree of habitat contamination (Hutton, 1981). Heavy metal pollution in birds can be assessed using different organs, tissues, feathers, pellets, feces, and eggs. Among these, feathers were widely used considering its feasibility than other methods involving tissue collection wherein the organism needs to be sacrificed or disturbed on its nest, while scouring of large forested area for pellets or fecal samples can be time and energy consuming. There are benefits of using feathers to quantify heavy metal contamination in avifauna (Movalli, 2000). Feathers are easy to collect, can be kept at room temperature, can be collected even from endangered species and harmless to the organism because feathers moult and eventually replaced with a new feather.

One of the ways by which heavy metal concentrations in the soil and feather can be determined is through X-ray fluorescence (XRF). XRF is a rapid and low-cost method used in the analysis of samples (Ulmanu et al., 2011). Hu et al. (2017) also stated that the XRF unlike the traditional laboratory methods of heavy metal analyses did not take much time and labor. Furthermore, the method is popular in different fields involving metals and its associated industries, study of earth and minerals, food industry and ecological management as well as reported by several authors in human health management (Al-Eshaikh & Kadachi, 2011)

According to Goede and de Bruin (1984), the heavy metal pollution studies using avian feathers began in 1966. Avian species may be contaminated through water, food, contact, respiration, and by deliberately taking in pebbles or soil particles and thus may reveal the ongoing pollution in the environment. Birds that are endemic or resident may provide clue on the level of heavy metal accumulation in the same area.

Heavy metal in avian species may come from the extraction of minerals and its associated activities like improper dumping of mine tailings and lack of technologies to regain chemicals used in its processing. Mine spill, open untreated tailings dam and dumping of contaminated mine gangue are among the causes of the heavy metal pollution in avian species, other wildlife, and in humans.

Study Area

Marinduque, an island province located 137 miles south of Manila is an ideal area to study heavy metals. The island located south of mainland Luzon only measures 172,700 hectares and is composed of six townships namely Boac, Gasan, Buenavista, Torrijos, Sta. Cruz, and Mogpog. In the municipality of Sta. Cruz, an eight-kilometer causeway was created from the mine tailings dumped in the vicinity of Barangay Ipil along Calancan Bay. The island province is also rich in biodiversity. In fact, seventy-five species of birds were observed in the island, of which, seventeen are endemic to the country and six are island endemic subspecies (Gonzalez & Dans, 2000). The possibility of detecting more species of birds in the said area is high as Sanchez (2015) captured some species that were not recorded in books such as by Kennedy et al. (2000).

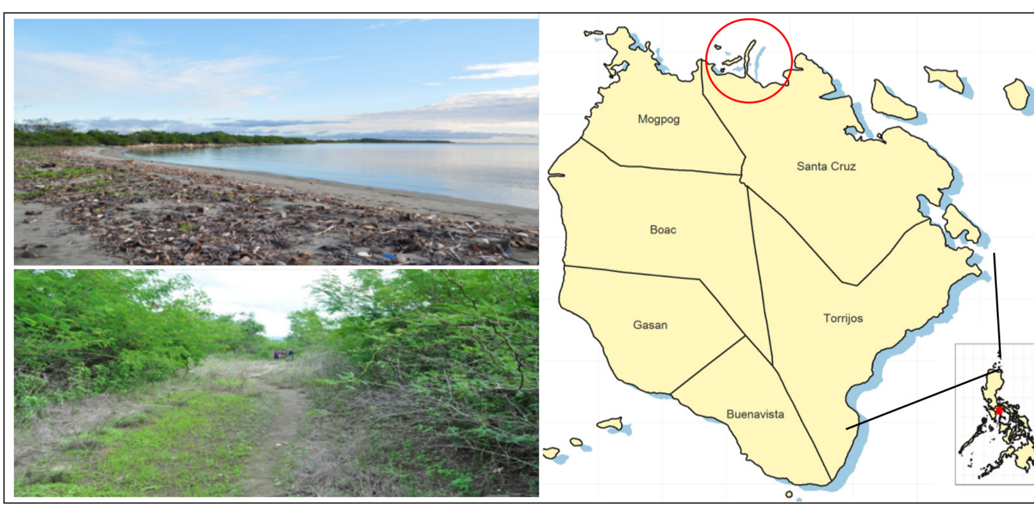

Figure 1. Map of Marinduque, its location (inset) and the study site (encircled red) (https://www.r-bloggers.com/creating-inset-map-with-ggplot2/)

This paper examined heavy metals in feathers of adult avian species captured from Barangay Ipil-Calancan Bay in the municipality of Sta. Cruz (Figure 1). This study sought to compare the present concentration of heavy metals in adult wild birds and pinpoint species that may be used as bioindicator or receptor species. Results of this study also provide baseline data on the level of heavy metal contamination in soil and birds and for comparison with future studies to monitor trends.

MATERIALS AND METHODS

All samples were collected with permission from the Department of Environment and Natural Resources. Birds were captured using mist nets and other indigenous traps. Collection of feathers from adult birds was done non-destructively. The feathers of the central tail, outermost primaries and secondaries of both wings, and some abdominal and scapular were pulled or cut using a sterile scissor. Newly grown or growing feathers were not removed to prevent bleeding and to ensure that only old plumages were utilized in this study. All feathers per individual bird were separately placed in a paper envelope coded with numbers corresponding with the list of those captured; the feathers contained in each envelope were then individually opened, cut into smaller pieces, packed in a polypropylene plastic, and sealed using a heat sealer. The soil samples, on the other hand, were collected from the 30cm and 60 cm depth in the site, air dried and kept in resealable plastic while in the field. After the fieldwork, the soil samples were air dried, packed and heat sealed. Each sealed polypropylene plastics containing the feathers and soils were then numbered accordingly. Lastly, the prepared samples were handed to the National Institute for Molecular Biology and Biotechnology of the University of the Philippines, Los Baños for XRF analysis to detect and quantify of the elements contained in the feathers and soils. Kruskal-Wallis and two tailed Pearson correlation were used to determine the differences and relationships of heavy metal concentrations between species and in soils.

RESULTS AND DISCUSSIONS

A total of 72 adult birds composed of 17 species and 11 families were captured. Of all the birds captured, only one was endemic, three endemic residents, and 10 were residents. Twelve of the species were common, three uncommon, and two fairly common. Only the range of the owl was not known as this owl had yet to be identified if it was a separate species but temporarily named for this study as Mantanani scops owl. Six species of wild doves or pigeons were netted, probably because the mangrove area and the trees that have grown in the causeway provided shelter and food. Of all the species of birds, the white collared kingfisher was relatively abundant (15.3%) and has the highest number of individuals captured. Based on relative abundance (RA, % of all species) the kingfisher was followed by zebra dove (11.1%), pied triller (9.7%), greater painted snipe (9.7%), yellow vented bulbul (8.3%), and island collared dove (6.9%) while the rest has four or less individuals captured (Table 1). Used nest mound of Tabon scrubfowls were also observed and three of this species were trapped.

Philippine duck (*Anas luzonica*), brahminy kite (*Haliastur indus intermedius*), purple heron (*Ardea purpurea*), osprey (*Pandion haliaetus melvillensis*), common moorhen (*Gallinula chloropus*), white breasted waterhen (*Amaurornis phoenicurus*), striated grassbird (*Megalurus palustris*), chestnut munia (*Lonchura malacca*), rails, Asian glossy starling (*Aplonis panayensis*), black-naped oriole (*Oriolus chinensis*), swiftlets, lowland white eye (*Zosterops meyeni),* and purple throated sunbird (*Nectarinia sperata*) were observed in the area.

Little is known on the accumulation of heavy metals and its present concentrations in birds from Marinduque Island especially in the tailings dumping site. Much of the studies on heavy metal pollution in the province were from sediments, fishes, corals, water, and other marine organisms. Twenty heavy metals out of the thirty-two elements identified by the XRF were used in this study. These heavy metals were As (Arsenic), Ba (Barium), Co (Cobalt), Cd (Cadmium), Cr (Chromium), Cu (Copper), Fe (Iron), Hg (Mercury), Mn (Manganese),

Table 1
List of wild avian species captured from the Barangay Ipil-Calancan Bay, Sta. Cruz, Marinduque Island, Philippines

Birds/Scientific name	**N=72**	**Range (Status)**	**RA**
Common emerald dove/*Chalcophaps indica*	4	Resident(Common)	5.5
Greater painted snipe/*Rostratula benghalensis*	7	Resident(Uncommon)	9.7
Island collared dove/*Streptopelia bitorquata*	5	Resident(Uncommon)*	6.9
Little heron/*Butorides striata carcinophilus*	2	Resident (Fairly common)	2.8
Mantanani scops owl/*Otus mantananensis*	3	? (Fairly common)*	4.2
Philippine bulbul/*Hypsipetes philippinus*	1	Endemic(Common)	1.4
Pied fantail/*Rhipidura nigritorquis*	3	Endemic(Common)[a]	4.2
Pied triller/*Lalage nigra*	7	Resident(Common)	9.7
Pink necked green pigeon/*Treron vernans*	3	Resident(Uncommon)	4.2
Spotted dove/*Streptopelia chinensis*	1	Resident(Common)	1.4
Tabon scrubfowl/*Megapodius cumingii pusillus*	3	Endemic Resident(Common)	4.2
White breasted woodswallow/*Artamus leucorhynchus*	3	Resident(Common)	4.2
White browed crake/*Porzana cinirea ocularis*	4	Endemic Resident(Common)	5.5
White collared kingfisher/*Halcyon chloris collaris*	11	Resident(Common)	15.3
White eared brown dove/*Phapitreron leucotis*	1	Endemic(Common)	1.4
Yellow vented bulbul/*Pycnonotus goiavier*	6	Endemic Resident(Common)	8.3
Zebra dove/*Geopelia striata*	8	Resident(Common)	11.1

Notes: All English and scientific names follow that of Kennedy et al. (2000), ***** = new record for the island province, [a] = lifted to species status (WBCP Checklist 2014), **?** = yet to be identified and classified

Mo (Molybdenum),Ni (Nickel), Pb (Lead), Sb (Antimony), Sn (Tin), Sc (Scandium), Se (Selenium), Sr (Strontium), Ti (Titanium), V (Vanadium), and Zn (Zinc).

The average of the three readings per individual of each species registered highest in Mantanani scops owl for Co, Pb, and Zn while Cr and Mn for Philippine bulbul. The yellow vented bulbul, Tabon scrubfowl, pied fantail, greater painted snipe, common emerald dove, and white collared kingfisher individually had the most elevated accumulation of Fe, Cu, Sc, Sr, and Se, respectively. Lowest average concentration of Fe and Cr was observed in pink necked green pigeon while Cu and Zn in white eared brown dove. Tabon scrubfowl, white collared kingfisher, little heron and spotted dove average for Mn, Sc, Se, and Sr were also respectively lowest (Figure 2).

The decelerating trend of heavy metals in wild avian feathers based on average concentration was Fe > Zn> Mn > Cu > Ti > Co > Cr > V> Mo > Sc > Pb > As > Se > Sr > Rb. On the other hand, Ba, Sb, Sn, Cd, Ni, and Hg were below the limit of detection. Sequentially, the highest to lowest concentration among the entire species of sampled birds was Tabon scrubfowl > Mantanani scops owl > Philippine bulbul > pied fantail > Mantanani Scops owl > Philippine bulbul > greater painted snipe > Mantanani scops owl > yellow vented bulbul > white collared kingfisher > common emerald dove. The Kruskal-Wallis test (Table 2) found highest concentrations among the sampled species especially with pied fantail for Cr and Cu and white browed crake for Fe and Sc. Strontium and Zn were also highly concentrated in significant amount with white collared kingfisher while Mn for little

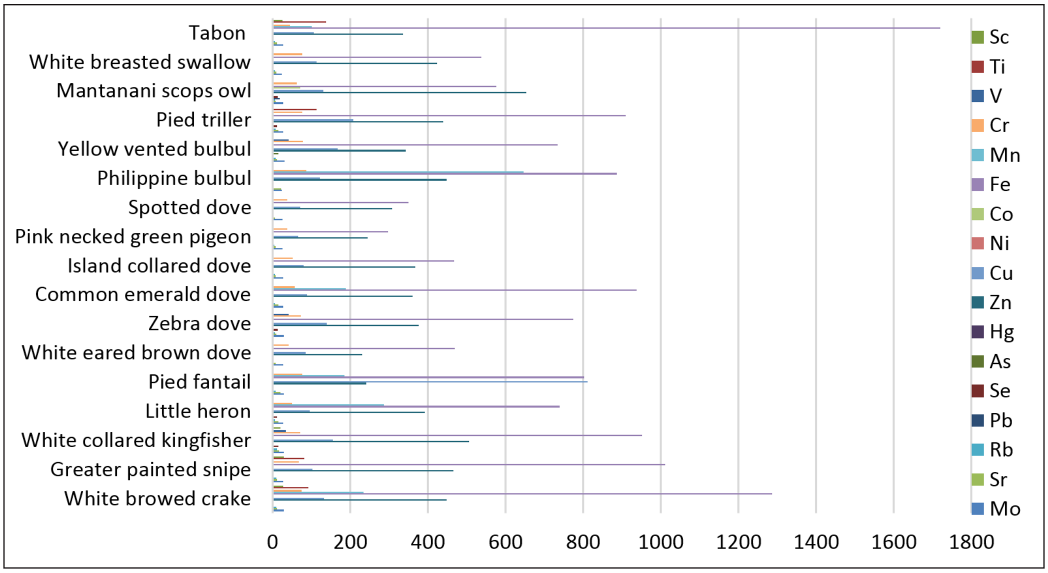

Figure 2. Heavy metal concentrations in wild avian species in Barangay Ipil-Calancan Bay, Sta. Cruz, Marinduque Island, Philippines

heron. Although Pb was high in all species and Se concentration in Mantanani scops owl was also elevated, both heavy metals were tested statistically trivial. The test also pointed out that most of the significant lowest accumulations were observed with Pink necked green pigeon (Cu, Fe, Sr, and Zn). Chromium was also observed to be concentrated at a significantly low amount in Tabon scrubfowl, including Sc and Mn with all birds except white browed crake and little heron.

The highest among the elements detected was Fe amounting to 1720.07mg/kg and found in Tabon scrubfowl. Iron also has the highest average concentrations in all sampled birds amounting to 791.17 mg/kg. This heavy metal can be elevated in most land-dwelling species as this essential element was also observed high by Sanchez (2015) with the red jungle fowl from the Marinduque Wildlife Sanctuary.

Cobalt, Pb and Zn were observed to be elevated in the Mantanani Scops owl. This owl prey upon small mammals, reptiles, large insects, and birds and most probably had acquired these metals from food. Elevated amount of Zn in raptorial birds was also observed in the study conducted by Nighat et al. (2013). According to Markowski et al. (2014), the presence of Cd can decrease the amount of Zn. On the other hand, increased Zn could prevent the harmful effect of Cd (Takekawa et al., 2002). The habit of deliberately taking in grit or small pebbles was thought to help in the digestion of food in owls may also add body burden of heavy metals. This habit was observed with the Philippine Scops owl *Otus megalotis megalotis*. The Philippine bulbul which can also be observed in the lowland and highland areas of the island province was detected to have much concentration of Cr and Mn. The yellow vented bulbul, a counterpart of the endemic bulbul was found to accumulate As. Both bulbul species may have accumulated the heavy metals from food and water in the area. Elevated concentrations of Cu were also observed with the pied fantail, an insectivorous and human refuse eater that occupies a wide range of habitat.

The statistical test regarding trophic level yielded highly significant concentrations of Cu (p = 0.000) with pied fantail together with pied triller and white breasted wood swallow. Significant concentrations of Sr, Mn, and Cr ($p = 0.000$, $p = 0.002$, and $p = 0.005$ respectively) were also observed with the insectivorous and frugivorous bulbuls that were sampled in this study. The concentrations of Fe in omnivores (greater painted snipe, Tabon scrubfowl, white browed crake and spotted dove) also tested highly significant ($p = 0.001$).Meanwhile, statistically significant ($p = 0.040$) amount of Se was observed with scops owl. Although the white collared kingfisher was observed to have the highest concentrations of Se among the sampled avian species, only Zn concentrations tested to be highly significant ($p = 0.000$).Increased concentrations of Se in the scops owl coincide with the findings of Ansara-Ross et al. (2015) where this element was found to be high in owls inhabiting near waterways. This suggests that birds living near waterways as well as those that largely get their food on tainted water are more prone to Se contamination. Sanchez (2015) also observed high Se concentrations in feathers of Indigo banded kingfisher from a different site.

Interestingly, the Pink-necked green pigeon inhabiting and foraging in the mangrove have four heavy metals (Cu, Fe, Sr, and Zn) of lowest amount among the group of birds from this site. The same elements were also observed low with the primary and secondary forest bird which is the tarictic hornbill (Sanchez, 2015). Both species rely on fruit bearing trees, palms, and berries. The pigeon might also forage in islets neighboring the tailings dumping site as well as in the nearby karst forest.

Of all the birds from this site having significant concentrations of heavy metals, the White collared kingfisher can be a good species to monitor bioaccumulation of Sr and Zn, the fantail for Cu and Cr, scops owl for Se and the crake for Fe and Sc (Table 2). The Yellow vented bulbul which was the only species detected to accumulate As on feathers can also be a receptor species for this element. The differences in the food item taken by each bird and the elimination processes may be the main factor in the accumulation of heavy metals on the feathers.

The descending order of the heavy metals detected on the soil samples was Fe > Ti > Cu > Mn > Sr > Ba > V > Zn > Cr > Sc > Sb > Pb > Sn> Rb > Mo > Cd and As. Selenium, Hg, Ni, and Co were below the limits of detection. The concentration of Pb, Zn, and Cr were higher than the Maximum Allowable Concentration (MAC) of substances in soil according to the USSR State Committee for Science and Technology while Mo, Ba, and Sn, Cd, and Cu, respectively, did not pass the category A, B, and C of the Dutch Soil Clean up Act (Beyer, 1990). The As concentration was

Table 2
Differences between heavy metal bioaccumulation (mg/kg) in various species of birds from Barangay Ipil-Calancan Bay, Sta. Cruz, Marinduque Island, Philippines

Heavy metals	**High species**	**Mean rank**	**Low species**	**Level of significance ($p = < 0.05$)**
Cr	Pied fantail	66.00±5.00	Tabon scrubfowl	0.000
Cu	Pied fantail	60.68±6.88	Pink necked green pigeon	0.000
Fe	White browed crake	60.25±2.67	Pink necked green pigeon	0.000
Mn	Little heron	50.50±33.0	All except Tabon scrubfowl Common emerald dove	0.000
Pb	All	34.5	All	1.000
Sc	White browed crake	50.50±33.5	All	0.002
Se	Mantanani scops owl	45.00±33.5	All except Pied triller	0.333
Sr	White-collared kingfisher	52.64±7.33	Pink necked green pigeon	0.001
Zn	White-collared kingfisher	16.67±3.33	Pink necked green pigeon	0.000

higher than the limits set by United States Environment Protection Agency (US EPA, 2002) and the New York State Department of Environmental Conservation (NYS DEC, 2006). Copper, Cr and Cd were also beyond the standards by the NYS DEC.

Using the two-tailed Pearson correlation, Mo from soil was found to be significantly correlated (0.01**, 0.05* level) with Fe**, Mn*, Cr*, V**, Ti* and Sb** while Zn with Sc**, Se with Sc** and Mn with Rb* and Sb* from collected feather samples. Studies of Van Der Merwe et al. (2011), Frantz et al. (2012), Torres et al. (2010), Markowski et al. (2014), Adout et al. (2007), and Levedeba (1997) found elevated heavy metal concentration such as Mn, Pb, Zn in bird's tissue were observed in contaminated sites, urban, farmland, and parkland than built up and rural areas. Contamination may imply correlation between feathers and soil in this present study as the sampling site was the tailing dumping area. Probably, the heavy metals in the feathers were from the environment and the contaminated food or water ingested in the area as all avian species subjected in this study were adult residents.

According to Theuerkauf et al. (2015) heavy metals are deleterious to diverse life forms. Essential heavy metals such as Se, Cr, Mn, Fe, Co, and Ni, may also be toxic in great amounts while Hg, Cd, and Pb are the non-essential and highly noxious. Detection of significant levels of As in feathers is hard because studies are limited (Burger et al., 2015). Arsenic was observed in a single species of bird burdened with 14.91 mg/kg. The As concentrations in the study by Burger et al. (2015) from shorebirds gathered were lower compared with this study. Iron and Zn detected in this study were lower with the concentrations in the Barn owl reported by Denneman and Douben (1993).

Generally, for the avian species, the trend in heavy metal concentration in this study was different with Abbasi et al. (2015) aside from Fe and Cu as consecutively being the highest. Cadmium and Ni concentrations were not detected in this study but detected with the research mentioned. Iron was the highest but followed by Cr instead of Cu and Ni was also present in studies by Theuerkauf et al. (2015). It was said that the low concentrations or below detection levels of Cd can be due to its non-accumulating capacity in feathers (Cochrane & Trust, 1996). Bond and Lavers (2010) cited that only 30% of Cd concentrations could be found on feathers. Meanwhile, the highest concentration of Fe in this study was higher than reported by Theuerkauf et al. (2015) but lower than that of Lervik (2012). Interspecies and intra-species variation in the concentrations of Fe in avian species was also observed by Goede and de Bruin (1984). These differences in concentrations of Fe between species and within the same species may also be detected with other heavy metals. According to Keith et al. (1989), differences in feather heavy metal concentrations are affected by the relative presence of the elements in the home range of each individual bird. Furthermore, factors such as gender, food preference, behavior, physiology, general health condition, and age among others, may play in this variation.

Heavy metal contamination may be through ingestion or contact with tainted matter. It was mentioned that proper cleaning of the feathers prior to analysis is vital when monitoring level of contamination via food (Denneman & Douben, 1993). But despite of rigid cleaning, some researchers like Denneman and Douben (1993) and Dmowski (1999) declared that most of the contamination could not be removed and washing could modify the quantity of the heavy metals endogenously present. On the other hand, washing hundreds of feathers and drying them was unsafe and may expose it to impurities other than those present at the study site. Keith et al. (1989) also noted that the plumage type, washing and evaluation procedures might influence variations in the element profiles. In this study, the feathers were not washed to determine the presence of contamination and samples were also subjected to XRF. Detection of heavy metals in feathers means contaminants are present in the environment and since all the sampled avian species were resident and endemic only proved that the contaminants were acquired from food and water in the habitat.

CONCLUSION

Quantification and understanding of heavy metals being a health and security threat to ecosystems, its components as well as to humans, is important in the decision making. The presence of heavy metals in feathers denotes that the contamination has reached higher trophic levels. Avian species either wild or domesticated provide nutrients to humans. Consumption of heavy metal laden food, water, and inhalation and direct contact with contaminated matters may imperil humans- its food and health security. This may also create a domino effect leading to disruption in the normal functioning of the ecosystem, biodiversity loss, and decreased ecosystem services.

The results of this study dictate that heavy metals abound in the area and affecting avian species. This may bring health and reproductive problems with avian species that may lead to imbalance in nature. Consequently, the problems that heavy metals may bring may greatly affect humans, their health, and livelihood. Some people especially those that hunt and or eat avian flesh may accumulate more of the noxious heavy metals.

To assess the effect of the heavy metals to humans in the area and to alleviate further health and economic problems it may bring, it is recommended for future studies to investigate the concentration and presence of the heavy metals in the edible parts and eggs of the free-range poultry and other food available for humans. The presence of heavy metals in the locals must also be investigated while planting of flora with high remediation capacity must be done. These can be done by the students of local educational institutions in partnership with the Department of Environment and Natural Resources and the Local Government Units. Replication of other past studies conducted in the area must also be done to monitor trends. The local people in this study site were participative however; sacrificing their poultry would require payment to compensate for their livelihood and other economic needs. On the other hand, for the results to reflect the true environmental condition, the poultry that would be studied must be free range, hatched, and raised in the same area.

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REFERENCES

Al-Eshaikh, M. A., & Kadachi, A. (2011). Elemental analysis of steel products using X-ray fluorescence (XRF) technique. *Journal of King Saud University-Engineering Sciences, 23*(2), 75-79. doi:10.1016/j.jksues.2011.03.002

Ansara-Ross, T. M., Ross, M. J., & Wepener, V. (2015). The use of feathers in monitoring bioaccumulation of metals and metalloids in the South African endangered African grass-owl (*Tyto capensis*). *Ecotoxicology, 22*(6), 1072–1083. http://doi.org/10.1007/s10646-013-1095-4

Abbasi, N. A., Jaspers, V. L. B., Chaudhry, M. J. I., Ali, S., & Malik, R. N. (2015b). Influence of taxa, trophic level, and location on bioaccumulation of toxic metals in bird's feathers: A preliminary biomonitoring study using multiple bird species from Pakistan. *Chemosphere, 120*(2015), 527–537. doi:10.1016/j.chemosphere.2014.08.054

Adout, A., Hawlena, D., Maman, R., Paz-Tal, O., & Karpas, Z. (2007). Determination of trace elements in pigeon and raven feathers by ICPMS. *International Journal of Mass Spectrometry, 267*(1-3), 109-116.

Beyer, W. N. (1990). Evaluating soil contamination. *Biological Rep., 90*(2), 1-25.

Bond, A. L., & Lavers, J. L. (2010). Trace elements concentrations in feathers of Flesh-footed shearwaters (*Puffinus carneipes*) from across their breeding range. *Archives of Environmental Contamination and Toxicology, 61*(2), 318-326. doi:10.1007/s00244-010-9605-3.

Burger, J., Tsipoura, N., Niles, L., Gochfeld M., Dey, A., & Mizrahi, D. (2015). Mercury, lead, cadmium, arsenic, chromium and selenium in feathers of shorebirds during migrating through Delaware Bay, New Jersey: Comparing the 1990s and 2011/2012. *Toxics, 3(1),* 63-74. doi:10.3390/toxics301006

Cochrane, J. F., & Trust, K. A. (1996). *Contaminant case report: Spectacled eider (Somateria fischeri) eggs and feathers from the Yukon-Kuskokwim Delta, Alaska*. Retrieved January 1, 2017, from http://ecos.fws.gov/ServCatFiles/reference/holding/21567

Denneman, W. D., & Douben, P. E. T. (1993).Trace metals in primary feathers of the Barn owl (*Tyto alba guttatus)* in the Netherlands*. Environmental Pollution, 82*(3)*,* 301-310.

Dmowski, K. (1999). Birds as indicators of heavy metal pollution: Review and examples concerning European species. *Acta Orn, 34,* 1-26.

Frantz, A., Pottier, M. A., Karimi, B., Corbel, H., Aubry, E., Haussy, C., & Castrec-Rouelle, M. (2012). Contrasting levels of heavy metals in the feathers of urban pigeons from close habitats suggest limited movements at a restricted scale. *Environmental Pollution, 168,* 23-28.

Goede, A. A., & De Bruin, M. (1984). The use of feather parts as a monitor for metal pollution. *Environmental Pollution Series B*, *8*(4), 281-298.

Gonzalez, J. C. T., & Dans, A. T. L. (2000). Birds and mammals of Calancan Bay, Marinduque Island, Philippines. *Sylvatrop, 10*(1&2), 145.

Hu, B., Chen, S., Hu, J., Xia, F., Xu, J., Li, Y., & Shi, Z. (2017) Application of portable XRF and VNIR sensors for rapid assessment of soil heavy metal pollution. *PLoS ONE, 12*(2), 1-11. e0172438. doi:10.1371/journal.pone.0172438

Hutton, M. (1981). Accumulation of heavy metals and selenium in three seabird species from United Kingdom. *Environmental Pollution Series A, Ecological and Biological, 26*(2), 129-145.

Keith, J. O., Okuno, I., & Bruggers, R. L. (1989). Identifying *Quelea* population by trace element analysis of feathers. In R. L. Bruggers & C. C. Elliott (Eds), *Quelea quelea-Africa's Bird Pest* (pp. 66-77). Oxford, UK: Oxford University Press.

Kennedy, R., Gonzales, P., Dickinson, R., Miranda, Jr., H., & Fisher, T. (2000). *A guide to the birds of the Philippines*. Oxford, UK: Oxford University Press.

Lebedeva, N. V. (1997). Accumulation of heavy metals by birds in the Southwest of Russia. *Russian Journal of Ecology, 28*(1), 41-46.

Lervik, K. (2012)**.** *Metal levels in blood and feather from incubating female common eiders (Somateria mollissima) in Svalbard***.** Retrieved January 1, 2017, fromhttp://www.diva-portal.org.

Markowski, M., Bańbura, M., Kaliński, A., Markowski, J., Skwarska, J., Wawrzyniak, J., Zieliński, P., & Bańbura, J. (2014). Spatial and temporal variation of lead, cadmium, and zinc in feathers of Great tit and Blue tit nestlings in Central Poland. *Archives of Environmental Contamination and Toxicology, 67*(4)*,* 507-518.

Movalli, P. A. (2000). Heavy metal and other residues in feathers of Laggar falcon *Falco biarmicus jugger* from six districts of Pakistan. *Environmental Pollution, 109*(2)*,* 267-275.

Nighat, S., Iqbal, S., Nadeem, M. S., T. Mahmood, S. I., & Shah. (2013). Estimation of heavy metal residues from feathers of Falconidae, Accipitridae, and Strigidae in Punjab, Pakistan. *Turkish Journal of Zoology, 37*(4)*,* 488-500.

New York State Department of Environmental Conservation. (2006). *New York state brownfield cleanup program development of soil cleanup objectives technical support document.* Retrieved January 1, 2017, from http://www.dec.ny.gov/chemical/34189.html

Pure Earth and Green Cross Switzerland. (2015). *2015 World's worst pollution problems. The new top six threats: A priority list for remediation.* Retrieved January 11, 2017, from http://www.worstpolluted.org/

Pure Earth and Green Cross Switzerland. (2016). *2015 World's worst pollution problems. The toxic beneath our feet*. Retrieved January 1, 2017, from http://www.worstpolluted.org/

Sanchez, M. S. (2015). *Bioaccumulation of heavy metals and prevalence of parasites in avian species on the landscape of Marinduque Island, Philippines* (Unpublished Doctoral thesis) University of the Philippines Los Baños, Philippines.

Takekawa, J. Y., Wainwright-De La Cruz, S. W., Holtem, R. L., & Yee, J. **(**2002). Relating body condition to inorganic contaminant concentrations of diving ducks wintering in coastal California. *Archives of Environmental Contamination of Toxicology, 42*(1)*,* 60-70.

Theuerkauf, J., Haneda, T., Sato, N. J., Ueda, K., Kuehn, R., Gula, R., & Watanabe, I. (2015). Naturally high heavy metal concentrations in feathers of the flightless Kagu *Rhynochetos jubatus*. *IBIS, 157*(1)*,* 177-180.

Torres, J., Foronda, P., Eira, C., Miquel, J., & Feliu, C. **(**2010). Trace element concentrations in *Raillietina micracantha* in comparison to its definitive host, the feral pigeon *Columba livia*in Sta Cruz de Tenerife (Canary Archipelago, Spain). *Archives of Environmental Contamination of Toxicology, 58*(1)*,* 176-182. doi:10.1007/s00244-009-9532-5

Ulmanu, M., Anger, I., Gamenţ, E., Mihalache, M., Neagu, E., & Ilie, L. (2011). *Rapid and low-cost determination of heavy metals in soil using X-ray portable instrument.* Retrieved January 5, 2017, from https://pdfs.semanticscholar.org/a012/29ad0074eb96bce1ffe480abcfc87902b057.pdf

United States of Environmental Protection Agency. (2002). *Supplemental guidance for developing soil screening levels for superfund sites.* Retrieved January 5, 2017, fromhttp://www.epa.gov/superfund/health/conmedia/soil/index.htm

Van Der Merwe, D., Carpenter, J. W., Neitfeld, J. C., & Miesner, J. F. (2011). Adverse health effects in Canada Geese (*Branta canadensis*) associated with waste from Zinc and Lead mines in the Tri-State mining district (Kansas, Oklahoma, and Missouri, USA). *Journal of Wildlife Diseases, 47*(3), 650-661.

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Review article

The Significance of Major Viral and Bacterial Diseases in Malaysian Aquaculture Industry

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ABSTRACT

Aquaculture is an upcoming industry in Malaysia as sustainable fish production with total production of 407,387 tonnes in 2016. As a growing industry, current practices have led to disease outbreaks which are highlighted as one of the major issues faced in aquaculture farms. Throughout the 20 years of disease reporting in Malaysia, several bacterial and viral diseases were found to persist in farms. In addition, emerging global diseases have also been detected in several farms. The consequence of these diseases were outbreaks led to huge economic losses. Eventually, the combination of persistent and emerging diseases creates a potential threat for the aquaculture industry and hence requires immediate attention. Therefore, this review summarises the major viral and bacterial pathogens of fish and crustaceans in the context of Malaysian aquaculture. It also considers the characteristics of disease and their impacts as well as the potential for future disease emergence in these aquatic animals as aquaculture industry continues to expand. This review serves as a platform for future directions in research in improved monitoring, detection and prevention as a step towards increasing biosecurity in Malaysian aquaculture.

Keywords: Aquaculture, bacterial, disease, Malaysia, viral

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INTRODUCTION

The Malaysian government has identified aquaculture as a major source for increasing fish production to meet domestic demand, accounting for 520,513 tonnes of total fish production in 2014. Increasing at a rate of 8% per annum, the aquaculture production target under National Agro-Food Policy (NAFP) was set at 794,000 tonnes by the end of 2020 (Department of Fisheries Malaysia [DOF], 2017). However, several problems arise from current aquaculture practices, such as the occurrence of diseases in the farms and environmental concerns (Ashley, 2007; Cao et al., 2007).

Disease occurrence and outbreaks gain precedence due to the potential to eradicate fish stocks within the farm, assisted by the ease of disease transmission through the water (Leung & Bates, 2013). Additionally, the combination of stressful conditions from high stocking density in farms and deteriorating environmental conditions further aggravate the occurrence and severity of diseases (Bowden, 2008). If diseases remain unresolved, farms are expected to incur loss of stocks, potentially disrupting operations of the facility and limiting growth in the aquaculture sector (Stentiford et al., 2012). Thus, disease management and biosecurity are key issues to be addressed by the aquaculture industry today (Tan et al., 2008). This paper aimed to review major viral and bacterial diseases in Malaysian aquaculture industry and propose directions for future studies and efforts on their prevention.

DISEASE MONITORING IN MALAYSIAN AQUACULTURE INDUSTRY

While disease outbreaks are common throughout the world, regions in Asia are often highlighted due to higher relative aquaculture production plus tropical conditions which increases risk of outbreaks (Leung & Bates, 2013). In many countries, awareness of aquaculture disease has increased investment in research and disease control programs, including Malaysia (Bondad-Reantaso et al., 2005).

In Malaysia, disease monitoring is conducted by the Fisheries Research Institute under the Department of Fisheries, as part of the Fish Health Surveillance Program. Reports are compiled by the National Fish Health Research Centre (NaFisH), which are then compiled by the Network of Aquaculture Centres in Asia-Pacific (NACA) and the Food and Agriculture Organisation of the United Nations (FAO) as active disease monitoring of aquaculture in the Southeast Asian region (Network for Aquaculture Centres in Asia-Pacific [NACA], 2018). In this review, the disease have been categorised as finfish viral diseases, crustacean viral diseases and bacterial diseases.

FINFISH VIRAL DISEASES

Herpesvirus Diseases

Herpesviruses in the family of *Alloherpesviridae* are predominantly pathogens of fish and amphibians. Key features of herpesviruses include a high level of host specificity and the ability to establish long term latency while interacting intricately with host defences (Hanson et al., 2011). Fish species affected by herpesviruses include koi (*Cyprinus carpio*), eels (*Anguilla* sp.), sturgeons (*Acipenser* sp.) and cod (*Gadus* sp.) (Hanson et al., 2011; Lepa & Siwicki, 2013). The koi herpesvirus and channel catfish virus were also detected in Malaysian aquaculture industry.

Koi Herpesvirus (KHV). KHV currently recognised as *Cyprinid herpesvirus 3* (CyHV3), is a major economic threat to common carp (*Cyprinus carpio*) and koi rearing industries worldwide. The key clinical signs include lethargy, loss of appetite and gill necrosis, usually resulting in death within two days of the onset of symptoms (Hedrick et al., 2000). Internally, severe inflammation can be observed in the gill, skin, kidney, liver, spleen, gastrointestinal system and brain of diseased fish (Pikarsky et al., 2004).

Up to date, KHV has been persistently detected in koi and common carp farms located in Perak, Negeri Sembilan and Selangor (NACA, 2018). However, no studies have been published detailing disease outbreaks attributed to the virus in Malaysia. Azila et al. (2012) detected CyHV3 in apparent healthy Malaysian koi stocks using ELISA-based techniques. This observation illustrates the importance of further surveillance of fish stocks to verify the status of the CyHV3 infection in Malaysian koi carps. In addition, KHV can remain latent in the leucocytes of healthy fish and act as a reservoir until rearing waters achieve a temperature range of 15-28°C, activating viral expression (Uchii et al., 2009). As such temperatures are prevalent across Malaysia, the carp aquaculture industry may be susceptible to disease outbreaks, especially in the event of temperature fluctuations (Azila et al., 2012).

Channel Catfish Virus (CCV). CCV also known as *Ictalurid herpesvirus 1*, is a cytopathic herpesvirus which mainly infects catfish and causes severe haemorrhageespecially in young fingerlings (Lepa & Siwicki, 2013). Other clinical symptoms include erratic swimming, exophthalmia, distended abdomen and haemorrhages at the base of fins (Camus, 2004). This disease was thought to be specific to the channel catfish (*Ictalurus punctatus*), but has been recently reported in striped catfish as well (Siti-Zahrah et al., 2013). Meanwhile, very few studies were conducted on the diseases affecting other catfishes cultured in Malaysia, including the African catfish (*Clarias gariepinus*), bagrid catfish (*Hemibagrus gracilis*) and striped catfish (*Pangasius hypophthalmus*) (DOF, 2016).

Detection of CCV in Malaysian catfish was first reported by Siti-Zahrah et al. (2013) who detected the virus in cage-cultured striped catfish, "ikan patin", resulting in 30-40% mortality rates throughout a two-year epidemiological study. However, CCV was not confirmed to be the cause of mortality due to the presence of bacterial-like lesions, which may have been as a result of *Aeromonas hydrophila* and *Flexibacter columnaris* infection.

Iridovirus Diseases

The family of *Iridoviridae* represent a group of large icosahedral viruses (120-200 nm in diameter) that have the ability to remain infectious without the viral envelope and may be released as naked particles following cell lysis (Chinchar et al., 2009). It was suggested that only genera *Lymphocystivirus*, *Ranavirus* and *Megalocytivirus* contribute to mortalities in fish with differing clinical symptoms. *Lymphocystiviruses* cause non-fatal, superficial dermal infections, while *Ranaviruses* and *Megalocytiviruses* cause serious systemic diseases with high mortality rates (Leu et al., 2013). Infection from *Ranaviruses* and *Megalocytiviruses* produce similar symptoms, such as dark skin colouration, abnormal swimming behaviour, skin lesion, haemorrhage and fin erosion (Whittington et al., 2010). While, *Megalocytiviruses* produce unique symptoms include the formation of hypertrophy in cells, which can lead to organ enlargement (Yanong & Waltzek, 2010).

Disease outbreaks caused by *Iridovirus* were first reported in 2009, which resulted in a cumulative mortality of 100% and loss of approximately RM 50,000 (NACA, 2018). An iridovirid disease outbreak was reported in grouper (*Epinephelus* sp.) cultures in Sabah, Malaysia, where *Megalocytivirus* was revealed to be the cause of mortality via phylogeny analysis and evidence of organ enlargement. While the actual cause of the outbreak was not verified, high water temperatures of approximately 28°C to 30°C had been reported to stimulate virus replication and were reported in the affected waters, possibly contributing to the outbreak (Razak et al., 2014).

Betanodavirus Diseases

Betanodavirus from the family of *Nodaviridae* are composed of a non-enveloped and icosahedral particle size of between 25 to 30 nm. This virus causes a major disease termed Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER), present mainly in groupers and seabass, but has also been reported in farmed cod (Hellberg et al., 2010). Clinical signs include loss of swimming orientation, lethargy, loss of appetite and bloated bellies. In addition, histological lesions also include cellular vacuolation, necrosis and neuronal degradation in the central nervous system (Vendramin et al., 2013). In Malaysia, *Betanodavirus* outbreaks have been reported in groupers, golden pompanos (*Trachinotus blochii*) and Asian seabass (*Lates calcarifer*).

Grouper. Groupers represent a major aquaculture species that can be threatened by the occurrence of VNN while also acting as a natural reservoir for the virus (Ma et al., 2012). Outbreaks have been studied across various countries and revealed high rates of mortality within a few days post infection (Kara et al., 2014; Vendramin et al., 2013).

Only one VNN outbreak occuring in Langkawi, Kedah has been reported by NACA (2018). The outbreak led to high mortalities of brown marbled groupers (*Epinephelus fuscoguttatus*) and giant groupers (*Epinephelus lanceolatus*), resulting in a loss of RM 131,400. The disease was identified via molecular methods by the NaFisH, Penang. A study by Ransangan et al. (2013) found that *Betanodavirus*-related mortalities might occur without displaying clinical symptoms in groupers which may result in fewer outbreaks categorised under VNN. In addition, persistence of *Betanodavirus* in the environment may also contribute to reoccurrence of outbreaks.

Asian Seabass. VNN is one of the key diseases which affects the Asian seabass aquaculture in Malaysia and is capable of vertical transmission. However, only one report of a disease outbreak in Malaysia has been published (Ransangan & Manin, 2010). Earlier studies of suspected outbreaks have been published, however, authors were unable to confirm whether the mortalities were a direct result of VNN or other causal agents (Ransangan et al., 1999).

A number of potential outbreaks have also occurred in Sabah, Malaysia but again have not been confirmed due to the lack of diagnostic facilities and expertise (Ransangan & Manin, 2010). An outbreak between July to October 2008 was confirmed to be caused by *Betanodavirus* using PCR, in addition to matching VNN clinical symptoms. Apart from that, the disease did not resurface in farms in recent years (NACA, 2018). On the other hand, larvae up to 50 days post-hatching were found to be more vulnerable, after which mortality rates reduced significantly (Hick et al., 2011). However, mortalities do not occur in older fishes and they remain as carriers for the virus and can potentially spread it via horizontal transmission (Gomez et al., 2004).

Golden Pompano. Golden pompano are cultured in Malaysian aquaculture on a small scale due to limitations in availability of seed and fingerlings (Food and Agriculture Organisation [FAO], 2007). Nevertheless, an outbreak of VNN on a golden pompano farm has been reported in Langkawi, which utilises deep-sea cages and suffered approximately 60% mortality rates within a week (Ransangan et al., 2011). However, the origin of infection could not be identified due to common reports of the virus in other Southeast Asian regions. Apart from that, no further outbreak was reported recently (NACA, 2018).

Tilapia Lake Virus Disease

The Tilapia Lake Virus Disease (TiLVD), also known as the syncytial hepatitis of tilapia (SHT) is a recently emerging disease which targets tilapias (*Oreochromis* sp.) and was first recognised in a freshwater lake in Israel (Dong et al., 2017). The causal agent is a novel segmented RNA virus called the Tilapia Lake Virus (TiLV) which was clasified as an Orthomyxo-like virus with negative-sense RNA but differs with the formation of syncytia, which is not a feature of orthomyxoviral infections (del-Pozo et al., 2017). Clinical symptoms include lethargy, skin erosion, hemorrhage and abdominal distension, which usually result in mortality within 10 days (Tattiyaponga et al., 2017). Meanwhile, histopathological symptoms show lesions found in multiple organs (Senapin et al., 2018; Tattiyapong et al., 2017). Although slight variations exists in the symptoms observed, such as necrosis of gastric glands (Ferguson et al., 2014), ocular lesions (Eyngor et al., 2014) and necrotic foci in kidney (Fathi et al., 2017), hepatitis was the most common histopathological feature found in TiLV outbreaks (Jansen et al., 2018).

Currently, TiLV is highlighted as an upcoming disease with its presence confirmed in Ecuador, Israel, Colombia, Egypt, Uganda, Tanzanian, India, Malaysia, and Thailand (Amal et al., 2018; Behera et al., 2018; Jansen et al., 2018; Mugimba et al., 2018; Senapin et al., 2018),while many other countries have also been listed to be at high risk of TiLVD (Dong et al., 2017). Several studies have developed methods of analysis, including suitable cell lines and virus detection with reverse-transcriptase PCR (Tsofack et al., 2017), as well as non-lethal methods for sampling (Liamnimitr et al., 2018).

Up to date, two outbreaks were reported in Malaysia occuring in tilapia farms in Kedah and Perlis (NACA, 2018). Meanwhile, an outbreak occuring in Selangor was reported by Amal et al. (2018), describing mass mortality occuring in a tilapia farm as a result of a co-infection of TiLV and *Aeromonas veronii*. The disease was confirmed via PCR sequencing analysis and histopathological detection of syncytial hepatitis cells suggesting potential synergystic co-infection of TiLV with other bacteria. Lastly, TiLV was also detected in wild tinfoil barb (*Barbonymus schwanenfeldii*) in Perlis (Abdullah et al., 2018). However, it was unconfirmed if the virus is infectious in tinfoil barb as virus culture was not performed. Nevertheless, the authors highlight potential risks of TiLV occuring in other freshwater fish, especially in the barbs family.

CRUSTACEANS VIRAL DISEASES

White Spot Syndrome

White spot syndrome (WSS) is a key disease in farmed prawns and shrimps, caused by the white spot syndrome virus (WSSV). WSSV is a non-occluded enveloped, rod-shaped double-stranded DNA virus with a tail-like appendage and can cause cumulative mortalities of 100% within two to seven days (Escobedo-Bonilla et al., 2008). The virus belongs to the genus *Whispovirus* in the family *Nimaviridae* (Sánchez-Paz, 2010). WSS was first reported in Malaysia in 1994 (Flegel, 1997) and has been observed in both tiger prawns (*Penaeus monodon*) and banana prawns (*Penaeus merguiensis*) in Malaysia (Wang et al., 2002).

A key symptom associated with WSS is the development of white spots on the exoskeleton and appendages, caused by the accumulation of cuticular substances on the inner surface of the cuticle following the destruction of cytoplasmic filaments post-infection (Rodríguez et al., 2003); however, this symptom is not diagnostic as it may also be caused by bacteria, high alkalinity and stress (Escobedo-Bonilla et al., 2008). Other symptoms include the pink-red colouration on the cephalothorax cuticle, reduction in feeding and increased lethargy, where infected shrimp swim slowly to the surface before sinking to the bottom to die (Sánchez-Martínez et al., 2007). Several internal organs also display destruction of tissue, accompanied by basophilic central inclusion bodies within the hypertrophied nuclei of cuticular epithelial and connective tissue cells (Escobedo-Bonilla et al., 2008). In addition, a yellow hypertrophied hepatopancreas may also develop due to haemolymph infiltration of the virus in the enlarged haemal sinuses and interstitial spaces (Sánchez-Martínez et al., 2007).

In Malaysia, several WSS outbreaks were investigated in a field study on shrimp farms from Penang, Kedah and Sarawak (Oseko, 2006). The author suggested that the poor environmental conditions on the farm led to the outbreak of this disease as previously reported in Thailand (Oseko, 2006). Two other studies also reported diseased tiger prawns obtained from farms along the coast of Peninsular Malaysia, indicating the occurrence of disease but this was not associated with any significant outbreak (Sahtout et al., 2001; Wang et al., 1995). While WSS has been commonly reported for tiger prawns, giant freshwater prawns (*Macrobrachium rosenbergii*), was found to be resistant to WSSV, possibly due to its innate immunity (Iwanaga & Lee, 2005).

White Tail Disease

White tail disease (WTD) is a common disease found in freshwater prawns and is caused by the *Macrobrachium rosenbergii* nodavirus (*Mr*NV) together with the extra small virus (XSV), where both are icosahedral and non-enveloped. The virus particles are commonly found in the cytoplasm of target cells and connective tissues, causing whitish colouration of the muscles starting from the tail to all prawn muscle. Eventually, the prawn shows abnormal behaviour, lethargy and anorexia, with mortality occuring within one to three days after the first clinical signs (Hameed & Bonami, 2012). Meanwhile, *Penaeus* prawns are not susceptible to this disease (Sudhakaran et al., 2006).

While outbreaks have not been reported, the *Mr*NV has been detected from the larvae of giant freshwater prawns randomly collected from hatcheries (Saedi et al., 2012). Detection of the virus was confirmed using RT-PCR and several samples were found to be positive for the virus despite showing no symptoms of WTD, which has also been reported previously (Widada et al., 2004). However, histological analysis of diseased prawns revealed progressive segmental myofibre degeneration of muscles and subsequent necrotic myopathy, both of which are associated with *Mr*NV infection (Saedi et al., 2012). As *Mr*NV has been detected in Malaysian freshwater prawns, future screening is vital to prevent the occurrence of outbreaks.

Infectious Hypodermal and Haematopoietic Necrosis Disease

Infectious hypodermal and haematopoietic necrosis disease (IHHNV) is a shrimp disease which causes high mortality rates in postlarvae and juvenile shrimps (Walker & Mohan, 2009). The causal agent, named after the disease, is a small icosahedral, non-enveloped virus from the family of *Parvoviridae* and contains a single-stranded linear DNA genome. Species affected by this disease includes blue shrimp (*Penaeus stylirostris*), whiteleg shrimp (*Penaeus vannamei*), tiger prawns, and giant freshwater prawns (Flegel, 2012).

The disease is fatal to blue shrimp and causes growth deformities in whiteleg shrimp and tiger prawn (Chayaburakul et al., 2005; Withyachumnarnkul et al., 2006). In larvae and juvenile giant freshwater prawns, high mortality rates occur while adults become carriers of the virus, which can be transmitted both horizontally and vertically (Hsieh et al., 2006). The clinical symptoms include large conspicuous eosinophilic intranuclear inclusions which occur in a range of organs, including the gills, nerve cords, stomach mucosa, antennal gland, and both hematopoietic and connective tissues. Infected prawns then become lethargic and swim on the surface before sinking to the bottom (Vega-Heredia et al., 2012).

In Malaysia, outbreaks of this disease has only been reported in recent years in farms located in Perak, Terengganu and Selangor, affecting giant freshwater prawn, tiger prawn and whiteleg shrimp (NACA, 2018). Prior to these outbreaks, several studies were conducted which detected the presence of IHHNV in wild prawn populations (Kua et al., 2011; Nita et al., 2012). In the study of Nita et al. (2012), wild giant freshwater prawns broodstocks from a river in Perak were screened and despite not displaying the usual symptoms, approximately 20% of the samples were found to be IHHNV positive. In contrast, another study from a river in Sarawak showed that, while clinical signs of disease were observed on giant freshwater prawns, but none of the samples were tested positive for IHHNV. The combination of these two studies suggest the presence of IHHNV in selected areas in Malaysia, highlighting the need for comprehensive screening of broodstocks before they are introduced into the hatchery (Nita et al., 2012).

FINFISH AND CRUSTACEAN BACTERIAL DISEASES

Vibriosis

Vibriosis is a major disease in aquaculture farms worldwide, caused by members from the genus *Vibrio*, which features Gram-negative, facultative anaerobic and rod shaped bacteria (Austin & Zhang, 2006). In addition to being an indigenous member of aquatic animals, *Vibrio* sp. also readily survive in the environment without being dependent on hosts, causing them to be a major issue in aquaculture (Ina-Salwany et al., 2018). Several notable species include *Vibrio harveyi*, *V. alginolyticus* and *V. anguillarum*. For Malaysian aquaculture, vibriosis has been reported in groupers, Asian seabass and crustaceans as described below:

Vibriosis in Fish. In fish, infection with *Vibrio* sp. commonly occurs in the intestinal epithelium when in contact with rearing water, potentially infecting and eliciting disease within a day (Verschuere et al., 2000). Penetration of host tissues is followed by the deployment of iron-sequestering systems and host cell damage via production of extra cellular products (Defoirdt, 2014). This leads to clinical symptoms such as septicaemia with haemorrhage at the base of fins, exophthalmia and cornea opacity in fishes. Fish exhibiting severe anaemia, as evidenced by pale gills, are at the moribund stage and are often anorexic. Oedematous lesions, predominantly centred on the hypodermis, were also often observed (Toranzo et al., 2005).

In the Malaysian aquaculture, vibriosis is a key disease faced by grouper farmers in Malaysia (Chuah, 2001). The disease was reported since the 1960s, where high mortality rates were observed in fish pens in Penang (Wong et al., 1979). Vibriosis was reported to occur mainly during the hatchery and grow-out phases; however, recent studies indicate that adults can also be affected (Nagasawa & Cruz-lacierda, 2004). Occurrence of vibriosis was also reported and found to be in combination with parasitic infections which occur at gills (Chuah, 2001). It is likely that the above correlates with the stress levels in groupers, such as higher temperatures which can lead to a reduction in immune responses and hence disease occurrence (Albert & Ransangan, 2013).

Meanwhile, for the Asian sea bass, an outbreak was reported in open net cages in Sabah, where the causal agent was identified as *V. harveyi* (Ransangan & Mustafa, 2009). The contributing cause of the outbreak was found to be the stressful conditions created from high stocking density of fish in net-cages. For adult fish, skin lesions, haemorrhage at the tips of fins and tails and eye opacity were the symptoms associated with vibriosis in Asian seabass. However, this is noted to be different from symptoms of vibriosis infection in other fish, which often present with gastroenteritis (Ransangan et al., 2012).

Vibriosis in Crustaceans. In crustaceans, infection with *Vibrio* sp. occurs mainly in the hepatopancreas - similar to other pathogenic bacteria. Infection of the digestive gland is eventually followed by the complete colonisation of the entire digestive tract. Clinical symptoms include severe damage to the hepatopancreas from inflammatory responses, leading to physiological imbalances and stress and eventually slower growth and mortality (Soonthornchai et al., 2015). Multiplication of bacteria on surfaces of shrimp, combined with poor nutritional and physiological conditions, may reduce the capacity of the shrimp to resist infection (Defoirdt et al., 2007). Luminous vibriosis is a form of the disease which occurs in high value crustaceans such as shrimps and tiger prawns. While this disease has been reported extensively in various countries practicing shrimp farming, very few studies report such outbreaks in Malaysia.

Acute Hepatopancreatic Necrosis Disease

Acute hepatopancreatic necrosis disease (AHPND) is a relatively new disease of shrimp, emerging in 2010 and was previously called 'early mortality syndrome' (EMS) due to mass mortality occurring within 10 days of post-stocking in a recently prepared pond (Tran et al., 2013). While the disease is generally caused by *Vibrio parahaemolyticus* carrying the toxin plasmids *PirA* and *PirB*, recent studies suggest *V. harveyi* strains may also elicit disease with the same plasmids. This disease has been reported to occur in shrimp farms worldwide, including Malaysia where the total economic loss from AHPND between 2011 to 2014 were estimated to reach US$ 0.49 billion (Kua et al., 2016).

A major clinical symptom of AHPND is the appearance of empty stomach and gut in tandem with a light-coloured, shrunken and atrophied hepatopancrease, which differs from a healthy dark-coloured brown hepatopancrease (Zorriehzahra & Banaederakhshan, 2015). Affected shrimp also often show soft shells, lethargy, muscle opacity and slow growth (Tran et al., 2013).

AHPND was first reported in Malaysia between late 2010 and early 2011, where several disease outbreaks occurring during the early stage of whiteleg shrimp culture due to *V. parahaemolyticus* infection (Kua et al., 2016). In 2011, high mortalities were reported to occur in shrimp farms throughout of Peninsular Malaysia, affecting the states of Perak, Penang, Kedah and Pahang. Eventually, samples from the states of Sabah, Sarawak, Terengganu, Johor and Melaka were also found positive for AHPND via histopathological analysis (Manan et al., 2015). While both whiteleg shrimp and tiger prawn were affected, infection rates were found to be higher in the former. High mortality rates were also documented in shrimp post larvae stages, juveniles and broodstocks. Nevertheless, prevalence of the disease has reduced throughout the years, from 50% in 2011 to 4% in 2015 (Kua et al., 2016).

Streptococcosis

Streptococcosis is a common disease which affects both Asian seabass, and hybrid tilapia and is caused by Gram-positive bacteria from the genus *Streptococcus. Streptococcus* sp. can also be found in the natural environment and a low bacteria load in water is sufficient to lead to mass mortality in fish (Agnew & Barnes, 2007). Major pathogens include *Streptococcus iniae* in Asian seabass and tilapia, and *S. agalactiae* in tilapia, which symptoms include erratic swimming, panophthalmitis, exophthalmos, corneal opacity and haemorrhage in the body cavity and ultimately resulted in mortality (Bromage & Owens, 2002). *Streptococcus* sp. have also been found to infect several organs, including the brain, blood, liver and kidney and liver of infected fishes (Abuseliana et al., 2011).

In Malaysia, streptococcosis has been reported only in tilapia and leading to 60-70% mortality in cage cultures located in Kenyir, Pedu and Pergau Lakes in 1997 (Siti-Zahrah et al., 2005). Several other studies have also reported on the mass mortalities and disease outbreaks caused by *S. agalactiae* (Abuseliana et al., 2011; Najiah et al., 2012). It was found that tilapia are more resistant towards water-based transmission of the pathogen when immersed in water containing 3 x 10^5

CFU/ml *S. agalactiae*, as no mortality was observed while clinical symptoms exhibited are limited to lethargy and loss of appetite (Abuseliana et al. 2011). Hence, it is unsure if this could lead to deterioration of health and ultimately susceptibility to the disease.

Meanwhile, Rahmatullah et al. (2017) reported on *S. iniae* isolated from tilapia cultured in Kenyir Lake. The fish showed abnormal swimming behaviour, haemorrhages were also found on pectoral, dorsal and caudal fins. The bacteria was isolated from brain, liver, kidney and eyeball. Experimental infection was also confirmed and the LD_{50} of the tilapia was 10^2 CFU/ml via IP injection, which was significantly lower bacteria concentration as opposed to other studies (Baums et al., 2013).

Overall, outbreaks of streptococcosis in the Malaysian aquaculture industry have been correlated with the intensification of tilapia aquaculture. Stressful conditions, including low water quality and high stocking density, can precipitate disease occurrence (Amal & Zamri-Saad, 2011). On the other hand, temperature fluctuations in the water due to dry seasons may also contribute to disease occurrence (Siti-Zahrah et al., 2004).

Motile Aeromonas Septicemia

Motile Aeromonas Septicaemia (MAS) is a disease linked to heavy mortalities in farmed and wild fishes globally (Harikrishnan & Balasundaram, 2005). The disease is caused by the genus *Aeromonas*, particularly *Aeromonas hydrophila* and *A. veronii* (Hamid et al., 2017). The virulence of Gram-negative *A. hydrophila* and *A. veronii* have been associated with virulence genes and haemolytic activity, allowing infections to occur when the host is under stress (Hamid et al., 2016). In Malaysia, *Aeromonas* sp. has been detected in giant freshwater prawns, tilapia and catfishes.

MAS is generally characterised by a wide range of symptoms caused by Aeronomad toxins (Laith & Najiah, 2013). Infected fish show abnormal swimming behaviour and aggregation on the water surface, with external symptoms including small surface lesions, local haemorrhages particularly in the gills and vent, ulcers, abscesses, exophthalmia, abdominal distensions (Hamid et al., 2017). Discolouration of catfish from normal gray to pinkish has also been associated as a symptom of infection from *Aeromonas* sp. (Anyanwu et al., 2015). Meanwhile, internal symptoms in fish include anemia, accumulation of ascitic fluid and ulcerative damage to the organs, notably the kidney and liver (Laith & Najiah, 2013).

Despite Aeromonas commonly reported as a major cause of mortality in the industry, only two cases of outbreaks were reported in Malaysia. The first case of mass mortality reported in a tilapia farm identified the causal agents to be a combination of *A. veronii* and the TiLV (Amal et al., 2018). The analysis of the symptoms, clinical signs and histopathological findings of affected fish also suggested that both pathogens acted synergistically, which has also been reported in Egypt and Thailand (Nicholson et al., 2017; Surachetpong et al., 2017). As TiLV is a major concern for the tilapia industry, the author cautions that such co-infections may further aggravate the issue and further studies should be conducted to understand the relationship of both pathogens (Amal et al., 2018).

Meanwhile, the second case reported the isolation and identification of *A. hydrophila* in diseased catfishes displaying common clinical and histological symptoms of MAS from a local farm (Laith & Najiah, 2013). The authors suggested that the virulence factor aerolysin and the presence of β-hemolysis in the bacteria may have played a role, which may have triggered the disease outbreak in addition to other factors including stress and changes in the environmental conditions (Laith & Najiah, 2013).

Piscine Tuberculosis

Piscine tuberculosis, also known as piscine mycobacteriosis, is caused by *Mycobacterium* sp., which are Gram-positive, pleomorphic, non-motile rod shaped bacterium found in soil and water. A wide range of species has been isolated from fishes, *Mycobacterium marinum*, *M. fortuitum* and *M. chelonae* are the only reported agents causing piscine mycobacteriosis (Gauthier & Rhodes, 2009).

As a chronic disease, piscine tuberculosis may not produce clinical symptoms, particularly in the early stages of infection. External clinical symptoms include scale loss and haemorrhagic lesions, abnormal behavior, spinal defects, emaciation, and ascites (Gauthier & Rhodes, 2009). Infected fish also show reduced food intake and weakened swimming one week prior to death (Swaim et al., 2006). Meanwhile, internal symptoms include enlargement of the spleen, kidney and liver, and characteristic grey or white nodules (granulomas) in internal organs (Toranzo et al., 2005). These granulomatous inflammation can be considered as a classic histopathological manifestation of chronic piscine mycobacteriosis, which are composed of concentric layers of epithelioid cells forming a discrete spherical lesions and is produced in multiple organs and tissues (Gauthier & Rhodes, 2009). While fish with chronic infections of mycobacteria may survive for up to eight weeks, acute infection usually leads to uncontrolled development of the pathogen and death of all fishes in within 16 days (van der Woude et al., 2014). Piscine tuberculosis outbreak has not been reported for aquaculture species in Malaysia despite being highlighted as a common bacterial disease in shrimp farms (Hashish et al., 2018).

Edwardsiellosis

Edwardsiellosis is a disease caused by genus of *Edwardsiella*, mainly *Edwardsiella tarda,* a Gram-negative, rod-shaped bacterium, which has been isolated from lakes, streams, seawater, mud and intestines of healthy aquatic animals (Choudhury et al., 2017; Mohanty & Sahoo, 2007). Edwardsiellosis causes spiralling movement and fish death with mouth agape and opercular flared, potentially due to anaemia and oxygen insufficiency. Other signs include lesions on skin, pale gills, tumefaction of the eye, excessive mucus secretion,scale erosion and ulcers (Xu & Zhang, 2014). Disease progression leads to the development of abscesses within the muscles of the flanks or caudal peduncle, which rapidly increases in size and further develops into large cavities filled with gas. Incission of these lesions lead to the emission of a foul odour from necrotic tissue remnants (Mohanty & Sahoo, 2007). Histologically, the lesions are characterised focal necrosis, often extending from muscle, haemopoietic tissue and liver parenchyma to perforate the abdominal wall (Choudhury et al., 2017).

So far, no reports have been published on *E. tarda* outbreaks in Malaysia. However, several studies report the isolation of *E. tarda* from fish. The first study reports bacteria isolation from diseased African catfish, tilapia, Asian swamp eel (*Monopterus albus*) and snakeskin gouramy (*Trichogaster pectoralis*) from commercial farms in Terengganu (Lee & Musa, 2008). The second study reports the isolation of *E. tarda* from an Asian seabass hatchery in Terengganu (Nadirah et al., 2012). Lastly, *E. tarda* was found to co-infect tilapia with *A. hydrophilla* in a farm in Kelantan, where fish displayed the symptoms of both MAS and edwardsiellosis (Lee & Wendy, 2017). Unfortunately, the specific cause of the disease was not investigated. One similarity shared between the studies reported heavy metal and antibiotic resistance in *E. tarda*, echoing concerns of the development of co-selection and cross-resistance in the bacteria community of the aquaculture environment in Malaysia (Lee & Wendy, 2017).

FUTURE DIRECTIONS AND PRIORITIES

Diseases continue to overwhelm the aquaculture industry, coupled with continuous intensification and expansion, eventually causing a constraint in economic and social developments in many countries, including Malaysia (Bondad-Reantaso et al., 2005). Several methods aimed at alleviating potential issues in the Malaysian aquaculture industry are proposed as follows:

Improving Disease Monitoring via Farmer Input

Farmers are ideal as a frontline for disease surveillance, especially via syndromic surveillance, which is described as “use of health-related information that may be indicative of a probability of change in the health of a population that merits further research or enables a timely impact assessment and action requirement” (Brugere et al., 2017). In brief, farmers can identify pending disease outbreaks via abnormal signs, such as patterns of swimming, poor growth and lack of food consumption (FAO, 2001). These signs could be verified with “point of care” surveillance tests which aspire to the ASSURED criteria, being affordable, sensitive, specific, user-friendly, robist, rapid, equipment-free and deliverable, which may be in forms of basic tests which can be used by untrained users while obtaining a robust result (Kettler et al., 2004). With immediate notification to authorities, action can be taken to remedy while also preventing disease outbreaks.

Improvement of Surveillance and Diagnostic Methods

Currently, the Malaysian Department of Fisheries carries out surveillance twice a year, while not much information is presented on diagnostics available (DOF, 2018). The development of risk-based surveillance (RBS), which emphasizes on the risk assessment methods and traditional design surveillance for appropriate and cost-effective data collection, could be applied in aquaculture (Stärk et al., 2006). An example for risk-assessment consideration is the identification of sources for pathogen exposure and transmission, such as via introduction of live fish directly onto farms or seasonal changes in temperature which contributes to disease spread. The identification of disease introduction and spread routes and subsequent allows biosecurity measures to be put in place to mitigate those risks (Oidtmann et al., 2013).

PCR is one of the most common methods applied for diagnostics of fish diseases in the Malaysian aquaculture industry. While its application in farms is a challenge due to the requirements of a thermocycler and technical knowledge. Several issues could be circumvented via use of loop mediated isothermal amplification (LAMP), which is similar to the PCR while being relatively equipment-free and robust (Adams & Thompson, 2011). Among the pathogens listed in this review, LAMP is currently shown to detect for CyHV-3, *Iridovirus*, *Betanodavirus*, WSSV, *Mr*NV and XSV, IHHNV, *V. parahaemolyticus, S. agalactiae,* and *E. tarda*. Alternatively, lateral flow devises (LFD) are also simple to use, cheap and rapid, which act via detection of specific antibodies of an infectious agent by its diffusion through porous substrate and the subsequent appearance of two coloured lines (Brugere et al., 2017). LFD are available for detection of WSSV and CyHV-3 (Sithigorngul et al., 2006; Vrancken et al., 2013).

Improvement and Enforcement of Biosecurity

In the Malaysian aquaculture industry, the biosecurity measures are categorised under the Official Analysis plan by the Department of Fisheries (DOF) which includes aquaculture farms, hatcheries, cages, ocean landings, export and import premises, as well as aquatic animal processing factories (DOF, 2018). The biosecurity plan aims to ensure that all farms operate in suitable locations while also adhering to biosecurity requirements. For aquatic animal health, surveillance is conducted bi-anually according to diseases outlined for that particular year according to the risk of diseases. The biosecurity plan can be improved further, such as through the development of contingency plans, and enforcement of record keeping and traceability.

Contingency plans reduce the impact of surprise as it includes familiarisation of the emergency situation through the assessment of risks, identification of uncertainties, definition of priorities and response strategies which may differ between aquaculture farms due to differences in equipment and management techniques, as well as the requirement of staff trained to act in an emergency (Georgiades et al., 2016). Such preparations ensure a farm's viability as they enable recovery from emergency situations while also reducing loss.

Enforcement of record keeping ensures product traceability, allowing identification of sources and input which led to disease, while also ensuring that other stock were not affected (Georgiades et al., 2016; Yanong, 2012). Additionally, the recording of environmental conditions and animal husbandry practices may also provide insights as to those conditions that lead to sub-optimal stock production or disease, forecasting potential issues and potentially preventing problems before they arise (Johansen, 2013; Meyers, 2010). While the degree of record keeping by individual farmers is uncertain, improving this practice as a form of biosecurity could benefit all farms in general with the information obtained.

CONCLUSION

In conclusion, this review has provided an overview of the types of viral and bacterial diseases detected in Malaysian aquaculture and their current status. Among the diseases highlighted, viral diseases are considered more significant due to persistence and the lack of means of recovery, whereas bacterial diseases are often a product of poor culture conditions. Instead of placing emphasis on the recovery from disease, prevention, through improved biosecurity measures, is highlighted as the key solution. These could also benefit environmental protection and provide sustainability if good aquaculture practices were implemented. Nevertheless, governing bodies could also play a role in the improvement of biosecurity, especially in smaller farms with lower capital. Ultimately, collective effort from all sides may effectively reduce the occurrences of disease, creating a long term benefit while preventing future economic losses to disease outbreaks.

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REFERENCES

Abdullah, A., Ramly, R., Ridzwan, M. S. M., Sudirwan, F., Abas, A., Ahmad, K., ... Kua, B. C. (2018). First detection of Tilapia Lake Virus (TiLV) in wild river carp (*Barbonymus schwanenfeldii*) at Timah Tasoh Lake, Malaysia. *Journal of Fish Diseases*, *41*(9), 1459-1462. doi: 10.1111/jfd.12843

Abuseliana, A. F., Daud, H. H. M., Aziz, S. A., Bejo, S. K., & Alsaid, M. (2011). Pathogenicity of *Streptococcus agalactiae* isolated from a fish farm in Selangor to juvenile red tilapia (*Oreochromis* sp.). *Journal of Animal and Veterinary Advances*, *10*(7), 914-919. doi: 10.3923/javaa.2011.914.919

Adams, A., & Thompson, K. D. (2011). Development of diagnostics for aquaculture: Challenges and opportunities. *Aquaculture Research*, *42*, 93-102. doi:10.1111/j.1365-2109.2010.02663.x

Agnew, W., & Barnes, A. C. (2007). *Streptococcus iniae*: An aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary Microbiology*, *122*(1-2), 1-15. doi: 10.1016/j.vetmic.2007.03.002

Albert, V., & Ransangan, J. (2013). Effect of water temperature on susceptibility of culture marine fish species to vibriosis. *International Journal of Research in Pure and Applied Microbiology*, *3*(3), 48-52.

Amal, M. N. A., & Zamri-Saad, M. (2011). Streptococcosis in tilapia (*Oreochromis niloticus*): A review. *Pertanika Journal of Tropical Agricultural Science*, *34*(2), 195-206.

Amal, M. N. A., Koh, C. B., Nurliyana, M., Suhaiba, M., Nor-Amalina, Z., Santha, S., ... Zamri-Saad, M. (2018). A case of natural co-infection of Tilapia Lake Virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality. *Aquaculture*, *485*, 12-16. doi: 10.1016/j.aquaculture.2017.11.019

Anyanwu, M. U., Chah, K. F., & Shoyinka, V. S. (2015). Evaluation of pathogenicity of motile *Aeromonas* species in African catfish. *International Journal of Fisheries and Aquatic Studies*, *2*(3), 93-98.

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science*, *104*(3-4), 199-235. doi: 10.1016/j.applanim.2006.09.001

Austin, B., & Zhang, X. H. (2006). *Vibrio harveyi*: A significant pathogen of marine vertebrates and invertebrates. *Letters in Applied Microbiology*, *43*(2), 119-124. doi: 10.1111/j.1472-765X.2006.01989.x

Azila, A., Way, K., Wood, G., Ainol, Y. M. Y., Kamisa, A., Norfauzana, M. A., ... Sabri, M. Y. (2012). Detection of koi herpesvirus (KHV) in *Cyprinius carpio* (Koi) stocks using Enzyme-Linked Immunosorbent Assay (ELISA). *Pertanika Journal of Tropical Agricultural Science*, *35*(1), 21-25.

Baums, C. G., Hermeyer, K., Leimbach, S., Adamek, M., Czerny, C. P., Hörstgen-Schwark, G., ... Steinhagen, D. (2013). Establishment of a model of *Streptococcus iniae* meningoencephalitis in Nile tilapia (*Oreochromis niloticus*). *Journal of Comparative Pathology*, 149(1), 94-102. doi: 10.1016/j.jcpa.2012.10.003

Behera, B. K., Pradhan, P. K., Swaminathan, T. R., Sood, N., Paria, P., Das, A., ... Parida, P. K. (2018). Emergence of Tilapia Lake Virus associated with mortalities of farmed Nile tilapia *Oreochromis niloticus* (Linnaeus 1758) in India. *Aquaculture*, *484*, 168-174. doi: 10.1016/j.aquaculture.2017.11.025

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., ... Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology*, *133*(3-4), 249-272. doi: 10.1016/j.vetpar.2005.07.005

Bowden, T. J. (2008). Modulation of the immune system of fish by their environment. *Fish and Shellfish Immunology*, *25*(4), 373-383. doi: 10.1016/j.fsi.2008.03.017

Bromage, E. S., & Owens, L. (2002). Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: Effects of different routes of exposure. *Diseases of Aquatic Organisms*, *52*(3), 199-205. doi: 10.3354/dao052199

Brugere, C., Onuigbo, D. M., & Morgan, K. L. (2017). People matter in animal disease surveillance: Challenges and opportunities for the aquaculture sector. *Aquaculture*, *467*, 158-169. doi: 10.1016/j.aquaculture.2016.04.012

Camus, A., (2004). *Channel catfish virus disease (SRAC Publication No. 4707)*. Retrieved November 29, 2018, from https://agrilifecdn.tamu.edu/fisheries/files/2013/09/SRAC-Publication-No.-4702-Channel-Catfish-Virus-Disease.pdf

Cao, L., Wang, W., Yang, Y., Yang, C., Yuan, Z., Xiong, S., & Diana, J. (2007). Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. *Environmental Science and Pollution Research-International*, *14*(7), 452-462. doi: 10.1065/espr2007.05.426

Chayaburakul, K., Lightner, D. V., Sriurairattana, S., Nelson, K. T., & Withyachumnarnkul, B. (2005). Different responses to infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* and *P. vannamei*. *Diseases of Aquatic Organisms*, *67*(3), 191-200. doi: 10.3354/dao067191

Chinchar, V. G., Hyatt, A., Miyazaki, T., & Williams, T. (2009). Family Iridoviridae: Poor viral relations no longer. In J. L. Van Etten (Ed.), *Lesser known large dsDNA viruses: Current topics in microbiology and immunology* (Volume 328, pp. 123-170). Berlin, Germany: Springer.

Choudhury, T. G., Vinay, T. N., Gupta, S. K., Gita, S., & Sarkar, B. (2017). Edwardsiellosis: An emerging disease in Indian aquaculture. *Aquaculture Times*, *3*(2), 38-40.

Chuah, T. T. (2001). Survey of grouper diseases in Malaysia. In M. G. Bondad-Reantaso, J. Humphrey, S. Kanchanakhan, & S. Chinabut (Eds.), *Report and Proceedings of APEC FWG Project 02/2000 "Development of a Regional Research Programme on Grouper Virus Transmission and Vaccine Development"* (pp. 38-40). Bangkok, Thailand: Asia Pacific Economic Cooperation (APEC), Fish Health Section of the Asian Fisheries Society (FHS/AFS), Aquatic Animal Health Research Institute (AAHRI), and Network of Aquaculture Centres in Asia Pacific (NACA).

Defoirdt, T. (2014). Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. *Reviews in Aquaculture*, *6*(2), 100-114. doi: 10.1111/raq.12030

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., & Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: Luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology*, *25*(10), 472-479. doi: 10.1016/j.tibtech.2007.08.001

del-Pozo, J., Mishra, N., Kabuusu, R., Cheetham, S., Eldar, A., Bacharach, E., ... Ferguson, H. W. (2017). Syncytial hepatitis of tilapia (*Oreochromis niloticus* L.) is associated with orthomyxovirus-like virions in hepathocytes. *Veterinary Pathology*, *54*(1), 164-170. doi: 10.1177/0300985816658100

Department of Fisheries Malaysia. (2016). *Annual fisheries statistics 2016*. Retrieved November 29, 2018, from https://www.dof.gov.my/index.php/pages/view/3049

Department of Fisheries Malaysia. (2017). *Malaysian fishing industry scenario*. Retrieved November 29, 2018, from https://www.dof.gov.my/index.php/pages/view/42

Department of Fisheries Malaysia. (2018). *Official Analysis*. Retrieved November 29, 2018, from https://www.dof.gov.my/index.php/pages/view/2341

Dong, H. T., Ataguba, G. A., Khunrae, P., Rattanarojpong, T., & Senapin, S. (2017). Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease. *Aquaculture*, *479*, 579-583. doi: 10.1016/j.aquaculture.2017.06.035

Escobedo-Bonilla, C. M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M. B., & Nauwynck, H. J. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *Journal of Fish Diseases*, *31*(1), 1-18. doi: 10.1111/j.1365-2761.2007.00877.x

Eyngor, M., Zamostiano, R., Tsofack, J. E. K., Berkowitz, A., Bercovier, H., Tinman, S.,

... Eldar, A. (2014). Identification of a novel RNA virus lethal to tilapia. *Journal of Clinical Microbiology*, *52*(12), 4137-4146. doi: 10.1128/JCM.00827-14

Fathi, M., Dickson, C., Dickson, M., Leschen, W., Baily, J., Muir, F., ... Weidmann, M. (2017). Identification of Tilapia Lake Virus in Egypt in Nile tilapia affected by 'summer mortality' syndrome. *Aquaculture*, *473*, 430-432. doi: 10.1016/j.aquaculture.2017.03.014

Ferguson, H. W., Kabuusu, R., Beltran, S., Reyes, E., Lince, J. A., & del Pozo, J. (2014). Syncytial hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): A case report. *Journal of Fish Diseases*, *37*(6), 583-589. doi: 10.1111/jfd.12142

Flegel, T. W. (1997). Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World Journal of Microbiology and Biotechnology*, *13*(4), 433-443. doi: 10.1023/A:1018580301578

Flegel, T. W. (2012). Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology*, *110*(2), 166-173. doi: 10.1016/j.jip.2012.03.004

Food and Agriculture Organisation. (2001). *Cage aquaculture: Regional reviews and global overview*. Retrieved November 29, 2018, from FAO Fisheries Technical Paper website: http://www.fao.org/docrep/005/y1679e/y1679e00.htm

Food and Agriculture Organisation. (2007). *Cage aquaculture: Regional reviews and global overview*. Retrieved November 29, 2018, from FAO Fisheries Technical Paper website: http://www.fao.org/docrep/010/a1290e/a1290e00.htm

Gauthier, D. T., & Rhodes, M. W. (2009). Mycobacteriosis in fishes: A review. *The Veterinary Journal*, *180*(1), 33-47. doi: 10.1016/j.tvjl.2008.05.012

Georgiades, E., Fraser, R., & Jones, B. (2016) *Options to strengthen on-farm biosecurity management for commercial and non-commercial aquaculture (Technical Paper No: 2016/47)*. Retrieved November 29, 2018, from http://researchrepository.murdoch.edu.au/id/eprint/42722/1/on-farm-aquaculture-biosecurity.pdf

Gomez, D. K., Sato, J., Mushiake, K., Isshiki, T., Okinaka, Y., & Nakai, T. (2004). PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *Journal of Fish Diseases*, *27*(10), 603-608. doi: 10.1111/j.1365-2761.2004.00577.x

Hameed, A. S., & Bonami, J. R. (2012). White tail disease of freshwater prawn, *Macrobrachium rosenbergii*. *Indian Journal of Virology*, *23*(2), 134-140. doi: 10.1007/s13337-012-0087-y

Hamid, N. H., Hassan, M. D., Sabri, M. M., Hasliza, A. H., Hamdan, R. H., Afifah, M. N., ... Fuad, M. M. (2017). Studies on pathogenicity effect of *Aeromonas hydrophila* infection in juvenile red hybrid tilapia *Oreochromis* sp. In D. Yulistiani, A. H. Wardhana, I. Inounu, S. Bahri, S. Iskandar, E. Wina ... D. L. Aquino (Eds.), *Proceedings of International Seminar on Livestock Production and Veterinary Technology* (pp. 532-539). Jakarta, Indonesia: IAARD Press.

Hamid, R., Ahmad, A., & Usup, G. (2016). Pathogenicity of *Aeromonas hydrophila* isolated from the Malaysian Sea against coral (*Turbinaria* sp.) and sea bass (*Lates calcarifer*). *Environmental Science and Pollution Research*, *23*(17), 17269-17276. doi: 10.1007/s11356-016-6655-8

Hanson, L., Dishon, A., & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses*, *3*(11), 2160-2191. doi: 10.3390/v3112160

Harikrishnan, R., & Balasundaram, C. (2005). Modern trends in *Aeromonas hydrophila* disease management with fish. *Reviews in Fisheries Science*, *13*(4), 281-320. doi: 10.1080/10641260500320845

Hashish, E., Merwad, A., Elgaml, S., Amer, A., Kamal, H., Elsadek, A., ... Sitohy, M. (2018). *Mycobacterium marinum* infection in fish and man: Epidemiology, pathophysiology and management; A review. *Veterinary Quarterly*, *38*(1), 35-46. doi: 10.1080/01652176.2018.1447171

Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J. V., Marty, G. D., Nordhausen, R. W., ... Eldar, A. (2000). A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *Journal of Aquatic Animal Health*, *12*(1), 44-57. doi: 10.1577/1548-8667(2000)012<0044:AHAWMM>2.0.CO;2.

Hellberg, H., Kvellestad, A., Dannevig, B., Bornø, G., Modahl, I., Haldorsen, R. N., ... Sindre, H. (2010). Outbreaks of viral nervous necrosis in juvenile and adult farmed Atlantic cod, *Gadus morhua* L., in Norway. *Journal of Fish Diseases*, *33*(1), 75-81. doi: 10.1111/j.1365-2761.2009.01092.x

Hick, P., Schipp, G., Bosmans, J., Humphrey, J., & Whittington, R. (2011). Recurrent outbreaks of viral nervous necrosis in intensively cultured barramundi (*Lates calcarifer*) due to horizontal transmission of *betanodavirus* and recommendations for disease control. *Aquaculture*, *319*(1-2), 41-52. doi: 10.1016/j.aquaculture.2011.06.036

Hsieh, C. Y., Chuang, P. C., Chen, L. C., Chien, M. S., Huang, K. C., Kao, H. F, ... Tsai, S. S. (2006). Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture*, *258*(1-4), 73-79. doi: 10.1016/j.aquaculture.2006.04.007

Ina-Salwany, M. Y., Al-Saari, N., Mohamad, A., Mursidi, F. A., Mohd-Aris, A., Amal, M. N. A., ... Zamri-Saad, M. (2018). Vibriosis in fish: A review on disease development and prevention. *Journal of Aquatic Animal Health*, *31*(1), 3-22. doi: 10.1002/aah.10045

Iwanaga, S., & Lee, B. L. (2005). Recent advances in the innate immunity of invertebrate animals. *BMB Reports*, *38*(2), 128-150. doi: 10.5483/BMBRep.2005.38.2.128

Jansen, M. D., Dong, H. T., & Mohan, C. V. (2018). *Tilapia Lake Virus: A threat to the global tilapia industry?*. Retrieved November 29, 2018, from https://onlinelibrary.wiley.com/doi/epdf/10.1111/raq.12254

Johansen, R. (2013). *Fish health report 2012*. Retrieved November 29, 2018, from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2013/fish-health-report-2012

Kara, H. M., Chaoui, L., Derbal, F., Zaidi, R., de Boisséson, C., Baud, M., & Bigarré, L. (2014). *Betanodavirus*-associated mortalities of adult wild groupers *Epinephelus marginatus* (Lowe) and *Epinephelus costae* (Steindachner) in Algeria. *Journal of Fish Diseases*, *37*(3), 273-278. doi: 10.1111/jfd.12020

Kettler, H., White, K., & Hawkes, S. J. (2004). *Mapping the landscape of diagnostics for sexually transmitted infections: Key findings and recommendations (No. TDR/STI/IDE/04.1)*. Retrieved November 29, 2018, from https://www.who.int/tdr/publications/tdr-research-publications/mapping-landscape-sti/en/

Kua, B. C., Ahmad, I., Siti Zahrah, A., Irene, J., Norazila, J., Nik Haiha, N., ... Teoh, T. P. (2016). Current status of acute hepatopancreatic necrosis disease (AHPND) of farmed shrimp in Malaysia. In R. V. Pakingking Jr., E. G. T. de Jesus-Ayson, & B. O. Acosta (Eds.), *Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical*

Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia (pp. 55-59). Tigbauan, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

Kua, B. C., Choong, F. C., Nita, M. K., Muhd Faizul, H. A. H., Bhassu, S., Imelda, R. R., & Mohammed, M. (2011). Screening of parasitic and IHHNV infections in wild giant freshwater prawn *Macrobrachium rosenbergii* from Rejang River at Kuching, Sarawak. *Tropical Biomedicine*, *28*(1), 85-89.

Laith, A. R., & Najiah, M. (2013). *Aeromonas hydrophila*: Antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). *Journal of Aquaculture Research and Development*, *5*(2), 1-7. doi: 10.4172/2155-9546.1000215

Lee, S. W., & Musa, N. (2008). Phenotyping, genotyping and whole cell protein profiling of *Edwardsiella tarda* isolated from cultured and natural habitat freshwater fish. *American-Eurasian Journal of Agricultural and Environmental* Sciences, *3*(5), 681-691.

Lee, S. W., & Wendy, W. (2017). Antibiotic and heavy metal resistance of *Aeromonas hydrophila* and *Edwardsiella tarda* isolated from red hybrid tilapia (*Oreochromis* spp.) coinfected with motile aeromonas septicemia and edwardsiellosis. *Veterinary World*, *10*(7), 803-807. doi: 10.14202/vetworld.2017.803-807

Lepa, A., & Siwicki, A. K. (2013). Fish herpesvirus diseases: A short review of current knowledge. *Acta Veterinaria Brno*, *81*(4), 383-389. doi: 10.2754/avb201281040383

Leu, J., Wu, M., & Chou, H. (2013). A comparative study between ranavirus and megalocytivirus infections in orange-spotted grouper (*Epienphelus coioides*). *Journal of Marine Science and Technology*, *21*, 58-64. doi: 10.6119/JMST-013-1220-2

Leung, T. L., & Bates, A. E. (2013). More rapid and severe disease outbreaks for aquaculture at the tropics: Implications for food security. *Journal of Applied Ecology*, *50*(1), 215-222. doi: 10.1111/1365-2644.12017

Liamnimitr, P., Thammatorn, W., U-thoomporn, S., Tattiyapong, P., & Surachetpong, W. (2018). Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture. *Aquaculture*, *486*, 75-80. doi: 10.1016/j.aquaculture.2017.12.015

Ma, H., Xie, J., Weng, S., Zhou, T., & He, J. (2012). Co-infection of megalocytivirus and viral nervous necrosis virus in a very severe mass mortality of juvenile orange-spotted groupers (*Epinephelus coioides*). *Aquaculture*, *358*, 170-175. doi: 10.1016/j.aquaculture.2012.06.032

Manan, H., Zhong, J. M. H., Othman, F., & Ikhwanuddin, M. (2015). Histopathology of the hepatopancreas of pacific white shrimp, *Penaeus vannamei* from none early mortality syndrome (EMS) shrimp ponds. *Journal of Fisheries and Aquatic Science*, *10*(6), 562-568. doi: 10.3923/jfas.2015.562.568

Meyers, T. (2010). *Regulation changes, policies and guidelines for Alaska fish and shellfish health and disease control (Regional Report Series 5J10-01)*. Retrieved November 29, 2018, from http://www.sf.adfg.state.ak.us/fedaidpdfs/RIR.5J.2010.01.pdf

Mohanty, B. R., & Sahoo, P. K. (2007). Edwardsiellosis in fish: A brief review. *Journal of Biosciences*, *32*(3), 1331-1344.

Mugimba, K. K., Chengula, A. A., Wamala, S., Mwega, E. D., Kasanga, C. J., Byarugaba, D. K., ... Mutoloki, S. (2018). Detection of Tilapia Lake Virus (TiLV) infection by PCR in farmed and wild Nile tilapia (*Oreochromis niloticus*) from Lake Victoria. *Journal of Fish Diseases*, *41*(8), 1181-1189. doi: 10.1111/jfd.12790

Nadirah, M., Najiah, M., & Teng, S. Y. (2012). Characterization of *Edwardsiella tarda* isolated from Asian seabass, *Lates calcarifer*. *International Food Research Journal*, *19*(3), 1247-1252.

Nagasawa, K., & Cruz-lacierda, E. R. (2004). *Diseases of cultured groupers*. Retrieved November 29, 2018, from https://repository.seafdec.org.ph/handle/10862/1702.

Najiah, M., Aqilah, N. I., Lee, K. L., Khairulbarryyah, Z., Mithun, S., Jalal, K. C. A., ... Nadirah, M. (2012). Massive mortality associated with *Streptococcus agalactiae* infection in cage-cultured red hybrid tilapia *Oreochromis niloticus* in Como River, Kenyir Lake, Malaysia. *Journal of Biological Sciences, 12*(8), 438-442. doi: 10.3923/jbs.2012.438.442

Network of Aquaculture Centres in Asia-Pacific. (2018). *Quarterly aquatic animal disease report (Asia-Pacific region)*. Retrieved November 10, 2018, from https://enaca.org/

Nicholson, P., Fathi, M. A., Fischer, A., Mohan, C., Schieck, E., Mishra, N., ... Jores, J. (2017). Detection of Tilapia Lake Virus in Egyptian fish farms experiencing high mortalities in 2015. *Journal of Fish Diseases*, *40*(12), 1925-1928. doi: 10.1111/jfd.12650

Nita, M. H., Kua, B. C., Bhassu, S., & Othman, R. Y. (2012). Detection and genetic profiling of infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in wild berried freshwater prawn, *Macrobrachium rosenbergii* collected for hatchery production. *Molecular Biology Reports*, *39*(4), 3785-3790. doi: 10.1007/s11033-011-1155-x

Oidtmann, B., Peeler, E., Lyngstad, T., Brun, E., Jensen, B. B., & Stärk, K. D. (2013). Risk-based methods for fish and terrestrial animal disease surveillance. *Preventive Veterinary Medicine*, *112*(1-2), 13-26. doi: 10.1016/j.prevetmed.2013.07.008

Oseko, N. (2006). Occurrence and prevention of white spot syndrome (WSSV) in Malaysia. In *Proceedings from the 32nd Edition* (pp. 422-431). Minamiise, Japan: National Research Institute of Aquaculture.

Pikarsky, E., Ronen, A., Abramowitz, J., Hutoran, M., Shapira, Y., Steinitz, M., ... Kotler, M. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *Journal of Virology*, *78*(17), 9544–9551. doi: 10.1128/JVI.78.17.9544-9551.2004

Rahmatullah, M., Ariff, M., Kahieshesfandiari, M., Daud, H. M., Zamri-Saad, M., Sabri, M. Y., ... Ina-Salwany, M. Y. (2017). Isolation and pathogenicity of *Streptococcus iniae* in cultured red hybrid tilapia in Malaysia. *Journal of Aquatic Animal Health*, *29*(4), 208-213. doi: 10.1080/08997659.2017.1360411

Ransangan, J., & Manin, B. O. (2010). Mass mortality of hatchery-produced larvae of Asian seabass, *Lates calcarifer* (Bloch), associated with viral nervous necrosis in Sabah, Malaysia. *Veterinary Microbiology*, *145*(1-2), 153-157. doi: 10.1016/j.vetmic.2010.03.016

Ransangan, J., & Mustafa, S. (2009). Identification of *Vibrio harveyi* isolated from diseased Asian seabass *Lates calcarifer* by use of 16S ribosomal DNA sequencing. *Journal of Aquatic Animal Health*, *21*(3), 150-155. doi: 10.1577/H09-002.1

Ransangan, J., Lal, T. M., & Al-harbi, A. H. (2012). Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*). *Malaysian Journal of Microbiology*, *8*(2), 104-115. doi: 10.21161/mjm.03512

Ransangan, J., Manin, B. O., Abdullah, A., Roli, Z., & Sharudin, E. F. (2011). *Betanodavirus* infection in golden pompano, *Trachinotus blochii*, fingerlings cultured in deep-sea cage culture facility in Langkawi, Malaysia. *Aquaculture*, *315*(3-4), 327-334. doi: 10.1016/j.aquaculture.2011.02.040

Ransangan, J., Manin, B. O., Lal, T. M. M., Sade, A., & Azila, A. (2013). *Betanodavirus* infection in marine fish aquaculture in Malaysia. *Research Journal of Animal, Veterinary and Fishery Sciences*, *1*(7), 10-15.

Ransangan, J., Mustafa, S., & Rahman, R. A. (1999). A first report of genetic erosion in hatchery stock of seabass (*Lates calcarifer*) in Sabah. *Borneo Science*, *5*, 77-89.

Razak, A. A., Ransangan, J., & Sade, A. (2014). First report of Megalocytivirus (*Iridoviridae*) in grouper culture in Sabah, Malaysia. *International Journal of Current Microbiology and Applied Sciences*, *3*(3), 896-909.

Rodríguez, J., Bayot, B., Amano, Y., Panchana, F., De Blas, I., Alday, V., & Calderón, J. (2003). White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure. *Journal of Fish Diseases*, *26*(8), 439-450. doi: 10.1046/j.1365-2761.2003.00483.x

Saedi, T. A., Moeini, H., Tan, W. S., Yusoff, K., Daud, H. M., Chu, K. B., ... Bhassu, S. (2012). Detection and phylogenetic profiling of nodavirus associated with white tail disease in Malaysian *Macrobrachium rosenbergii* de Man. *Molecular Biology Reports*, *39*(5), 5785-5790. doi: 10.1007/s11033-011-1389-7

Sahtout, A. H., Hassan, M. D., & Shariff, M. (2001). DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). *Diseases of Aquatic Organisms*, *44*(2), 155-159. doi: 10.3354/dao044155

Sánchez-Martínez, J. G., Aguirre-Guzmán, G., & Mejía-Ruíz, H. (2007). White spot syndrome virus in cultured shrimp: A review. *Aquaculture Research*, *38*(13), 1339-1354. doi: 10.1111/j.1365-2109.2007.01827.x

Sánchez-Paz, A. (2010). White spot syndrome virus: An overview on an emergent concern. *Veterinary Research*, *41*(6), 43. doi: 10.1051/vetres/2010015

Senapin, S., Shyam, K. U., Meemetta, W., Rattanarojpong, T., & Dong, H. T. (2018). Inapparent infection cases of Tilapia Lake Virus (TiLV) in farmed tilapia. *Aquaculture*, *487*, 51-55. doi: 10.1016/j.aquaculture.2018.01.007

Sithigorngul, W., Rukpratanporn, S., Pecharaburanin, N., Longyant, S., Chaivisuthangkura, P., & Sithigorngul, P. (2006). A simple and rapid immunochromatographic test strip for detection of white spot syndrome virus (WSSV) of shrimp. *Diseases of Aquatic Organisms*, *72*(2), 101-106. doi: 10.3354/dao072101

Siti-Zahrah, A., Misri, S., Padilah, B., Zulkafli, R., Kua, B. C., Azila, A., & Rimatulhana, R. (2004). Pre-disposing factors associated with outbreak of Streptococcal infection in floating cage-cultured tilapia in reservoirs. In *7th Asian Fisheries Forum 2004, The Triennial Meeting of The Asian Fisheries Society* (p. 129). Bayan Lepas, Malaysia: WorldFish Center.

Siti-Zahrah, A., Padilah, B., Azila, A., Rimatulhana, R., & Shahidan, H. (2005). Multiple streptococcal species infection in cage-cultured red tilapia, but showing similar clinical signs. In M. G. Bondad-Reantaso, C. V. Mohan, M. Crumlish, & R. P. Subasinghe (Eds.), *Proceedings of the Sixth Symposium on Diseases in Asian Aquaculture* (pp. 313-320). Manila, Phillipines: Fish Health Section, Asian Fisheries Society.

Siti-Zahrah, A., Zamri-Saad, M., Firdaus-Nawi, M., Hazreen-Nita, M. K., & Nur-Nazifah, M. (2013). Detection of channel catfish virus in cage-cultured *Pangasius hypophthalmus* (Sauvage, 1878) in Malaysia. *Journal of Fish Diseases*, *37*(11), 981-983. doi: 10.1111/jfd.12185

Soonthornchai, W., Chaiyapechara, S., Jarayabhand, P., Söderhäll, K., & Jiravanichpaisal, P. (2015). Interaction of *Vibrio* spp. with the inner surface of the digestive tract of *Penaeus monodon*. *PLOS One*, *10*(8), e0135783. doi: 10.1371/journal.pone.0135783

Stärk, K. D., Regula, G., Hernandez, J., Knopf, L., Fuchs, K., Morris, R. S., & Davies, P. (2006). Concepts for risk-based surveillance in the field of veterinary medicine and veterinary public health: Review of current approaches. *BMC Health Services Research*, *6*(1), 20. doi: 10.1186/1472-6963-6-20

Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., ... Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology*, *110*(2), 141-157. doi: 10.1016/j.jip.2012.03.013

Sudhakaran, R., Syed Musthaq, S., Haribabu, P., Mukherjee, S. C., Gopal, C., & Hameed, A. S. (2006). Experimental transmission of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and extra small virus (XSV) in three species of marine shrimp (*Penaeus indicus*, *Penaeus japonicas* and *Penaeus monodon*). *Aquaculture*, *257*(1-4), 136-141. doi: 10.1016/j.aquaculture.2006.02.053

Surachetpong, W., Janetanakit, T., Nonthabenjawan, N., Tattiyapong, P., Sirikanchana, K., & Amonsin, A. (2017). Outbreaks of Tilapia Lake Virus infection, Thailand, 2015–2016. *Emerging Infectious Diseases*, *23*(6), 1031-1033. doi: 10.3201/eid2306.161278

Swaim, L. E., Connolly, L. E., Volkman, H. E., Humbert, O., Born, D. E., & Ramakrishnan, L. (2006). *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infection and Immunity*, *74*(11), 6108-6117. doi: 10.1128/IAI.00887-06

Tan, Z., Komar, C., & Enright, W. J. (2008). Health management practices for cage aquaculture in Asia - A key component for sustainability. In Y. Yang, S. Silva, & Y. Zhou (Eds.), *The Proceedings of the 2nd International Symposium on Cage Aquaculture in Asia (CAA2)* (pp. 17-33). Manila, Philippines: Asian Fisheries Society.

Tattiyapong, P., Dachavichitleada, W., & Surachetponga, W. (2017). Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.). *Veterinary Microbiology*, *207*, 170-177. doi: 10.1016/j.vetmic.2017.06.014

Toranzo, A. E., Magariños, B., & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, *246*(1-4), 37-61. doi: 10.1016/j.aquaculture.2005.01.002

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms*, *105*(1), 45-55. doi: 10.3354/dao02621

Tsofack, J. E. K., Zamostiano, R., Watted, S., Berkowitz, A., Rosenbluth, E., Mishra, N., ... Bacharach, E. (2017). Detection of Tilapia Lake Virus in clinical samples by culturing and nested reverse transcription-PCR. *Journal of Clinical Microbiology*, *55*(3), 759-767. doi: 10.1128/JCM.01808-16

Uchii, K., Matsui, K., Iida, T., & Kawabata, Z. (2009). Distribution of the introduced *cyprinid herpesvirus 3* in a wild population of common carp, *Cyprinus carpio* L. *Journal of Fish Diseases*, *32*(10), 857-864. doi: 10.1111/j.1365-2761.2009.01064.x

van der Woude, A. D., Stoop, E. J., Stiess, M., Wang, S., Ummels, R., van Stempvoort, G., ... Luirink, J. (2014). Analysis of SecA2-dependent substrates in *Mycobacterium marinum* identifies protein kinase G (PknG) as a virulence effector. *Cellular Microbiology*, *16*(2), 280-295. doi:10.1111/cmi.12221

Vega-Heredia, S., Mendoza-Cano, F., & Sánchez-Paz, A. (2012). The infectious hypodermal and haematopoietic necrosis virus: A brief review of what we do and do not know. *Transboundary and Emerging Diseases*, *59*(2), 95-105. doi: 10.1111/j.1865-1682.2011.01249.x

Vendramin, N., Patarnello, P., Toffan, A., Panzarin, V., Cappellozza, E., Tedesco, P., ... Cattoli, G. (2013). Viral Encephalopathy and Retinopathy in groupers (*Epinephelus* spp.) in southern Italy: A threat for wild endangered species?. *BMC Veterinary Research*, *9*(1), 20. doi: 10.1186/1746-6148-9-20

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*, *64*(4), 655-671. doi: 10.1128/MMBR.64.4.655-671.2000

Vrancken, R., Boutier, M., Ronsmans, M., Reschner, A., Leclipteux, T., Lieffrig, F., ... Goris, N. (2013). Laboratory validation of a lateral flow device for the detection of CyHV-3 antigens in gill swabs. *Journal of Virological Methods*, *193*(2), 679-682. doi: 10.1016/j.jviromet.2013.07.034

Walker, P. J., & Mohan, C. V. (2009). Viral disease emergence in shrimp aquaculture: Origins, impact and the effectiveness of health management strategies. *Reviews in Aquaculture*, *1*(2), 125-154. doi: 10.1111/j.1753-5131.2009.01007.x

Wang, C. H., Lo, C. F., Leu, J. H., Chou, C. M., Yeh, P. Y., Chou, H. Y., ... Kou, G. H. (1995). Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Diseases of Aquatic Organisms*, 23(3), 239-242. doi: 10.3354/dao023239

Wang, Y. T., Liu, W., Seah, J. N., Lam, C. S., Xiang, J. H., Korzh, V., & Kwang, J. (2002). White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. *Diseases of Aquatic Organisms*, *52*(3), 249-259. doi: 10.3354/dao052249

Whittington, R. J., Becker, J. A., & Dennis, M. M. (2010). Iridovirus infections in finfish - Critical review with emphasis on ranaviruses. *Journal of Fish Diseases*, *33*(2), 95-122. doi: 10.1111/j.1365-2761.2009.01110.x

Widada, J. S., Richard, V., Shi, Z., Qian, D., & Bonami, J. R. (2004). Dot-blot hybridization and RT-PCR detection of extra small virus (XSV) associated with white tail disease of prawn *Macrobrachium rosenbergii*. *Diseases of Aquatic Organisms*, *58*(1), 83-87. doi: 10.3354/dao058083

Withyachumnarnkul, B., Chayaburakul, K., Lao-Aroon, S., Plodpai, P., Sritunyalucksana, K., & Nash, G. (2006). Low impact of infectious hypodermal and hematopoietic necrosis virus (IHHNV) on growth and reproductive performance of *Penaeus monodon*. *Diseases of Aquatic Organisms*, *69*(2-3), 129-136. doi: 10.3354/dao069129

Wong, S. Y., Ong B., & Chua, T. E. (1979). Isolation, identification of causative agent of "red boil disease" in grouper (*Epinephelus salmoides*) and its possible control by vaccination. In D. A. Turnbull (Ed.), *Proceedings of the International Workshop on Pen Cage Culture of Fish* (pp 81-87). Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

Xu, T., & Zhang, X. H. (2014). *Edwardsiella tarda*: An intriguing problem in aquaculture. *Aquaculture*, *431*, 129-135. doi: 10.1016/j.aquaculture.2013.12.001

Yanong, R. P. (2012). *Biosecurity in aquaculture, Part 2: Recirculating aquaculture systems*. Retrieved November 29, 2018, from http://agrilifecdn.tamu.edu/fisheries/files/2013/09/

SRAC-Publication-No.-4708-Biosecurity-in-Aquaculture-Part-2-Recirculating-Aquaculture-Systems.pdf

Yanong, R. P. E. & Waltzek, T. B. (2010). *Megalocytivirus infections in fish, with emphasis on ornamental species*. Retrieved November 29, 2018, from University of Florida Institute of Food and Agricultural Sciences Extensions website: http://edis.ifas.ufl.edu/fa182

Zorriehzahra, M. J., & Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as new emerging threat in shrimp industry. *Advances in Animal and Veterinary Sciences*, *3*(2S), 64-72. doi: 10.14737/journal.aavs/2015/3.2s.64.72

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Javanese Medaka (*Oryzias javanicus* Bleeker, 1854) as Potential Model Organism for Aeromoniasis and Vibriosis Study in Fish

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ABSTRACT

Javanese medaka is a small tropical fish that has been widely used as a test organism in ecology, toxicology and ecotoxicology, but less in fish-pathogen related study. This study evaluates the capability of Javanese medaka as an alternative model organism for aeromoniasis and vibriosis study in fish. Javanese medakas were infected with different concentrations of *Aeromonas hydrophila*, *Vibrio alginolyticus* and *V. harveyi* via intraperitoneal (IP) and immersion (IMM) routes. Following the infections, the $LD_{50\text{-}240h}$ of all bacteria to Javanese medaka were observed lower in IP, compared with IMM route. In IP route, the $LD_{50\text{-}240h}$ ranged from 2.1×10^4 CFU/mL for *V. harveyi* to 2.5×10^7 CFU/mL for *A. hydrophila*. However, for IMM route, the range was from 6.6×10^7 CFU/mL for *V. harveyi* to 1.1×10^9 CFU/mL for *V. alginolyticus*. The clinical signs, gross lesions and histopathological changes of Javanese medakas infected by either IP or IMM routes of all the pathogens were

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similarly observed in the real host of the pathogen. Moreover, in IP route, most of the fish mortalities (88.9%) were observed within ≤ 120 hours post infection (hpi), while for IMM, most of the mortalities (50.6%) were witnessed beyond 120 hpi, indicating the acute infection for IP compared to IMM route. This study highlights the potential utilization of Javanese medaka as another valuable *in vivo* model organism for bacterial diseases study in fish.

Keywords: Aquaculture, fish diseases, Javanese medaka, test organism

INTRODUCTION

Javanese medaka (*Oryzias javanicus* Bleeker, 1854) is a small tropical fish that has the potential to be used as a test fish, since it showed characteristics similar to the established laboratory fishes (Imai et al., 2007). This species is also used to represent the fresh and marine water fishes, due to its ability to occupy fresh, brackish and saltwater (Inoue & Takei, 2002). Moreover, Javanese medaka has also been widely used as a test organism in ecology, toxicology and ecotoxicology (Aziz et al., 2017; Ferdaus et al., 2018; Ismail & Yusof, 2011; Salleh et al., 2017; Yusof et al., 2012, 2014). Javanese medakas are year-round available, fast in growth rate, hardy to poor water quality, having a short life span and life cycle, easily identified and culturable in the laboratory, and have a wide geographical range (Naruse et al., 2011). This species is widely distributed in Indonesia, Western Borneo, Singapore, Thailand (Magtoon & Termvidchakorn, 2009; Termvidchakorn & Magtoon, 2008), and along the coastal areas of Peninsular Malaysia (Yusof et al., 2012). However, Javanese medaka is less utilized as a test organism for study of bacterial infection in fish compared with zebrafish (*Danio rerio*) (Bakar et al., 2017; Liu et al., 2015; Neely et al., 2002; Patterson et al., 2012; Phelps et al., 2009; Rodríguez et al., 2008; Schmidt et al., 2017).

Intensification of aquaculture industry has led to various disease outbreaks, especially due to bacterial infections (Ismail et al., 2016, 2017; Toranzo et al., 2005). *Aeromonas hydrophila*, *Vibrio alginolyticus* and *V. harveyi* are among the important pathogens in fresh and marine water fish culture (Haenen et al., 2014; Hossain et al., 2014; Norhariani et al., 2019). Infections of *A. hydrophila*, *V. alginolyticus* and *V. harveyi* are commonly reported in major cultured fishes around the world, such as catfish (*Clarias* spp.), Asian seabass (*Lates calcarifer*) and hybrid grouper (*Epinephelus* spp.) respectively, and cause massive economic losses to the industry worldwide (Azila et al., 2017; Hossain et al., 2014; Ina-Salwany et al., 2018; Nurliyana et al., 2019a, b, c, d; Sharma et al., 2013; Zhu et al., 2017).

In the present study, we describe for the capability of Javanese medaka as an alternative model organism for aeromoniasis and vibriosis study in fish. We demonstrate that Javanese medaka is susceptible following intraperitoneal (IP) and immersion (IMM) challenges by *A. hydrophila*, *V. alginolyticus* and *V. harveyi*. These results constitute a proof of concept study for the use of Javanese medaka in understanding bacterial disease study.

MATERIALS AND METHODS

Ethic Statements

The fish were sampled, handled and sacrificed according to the methods approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia (AUP No.: R006/2016). All experiments were performed according to the mentioned guidelines and regulations.

Fish Collection and Acclimatization

Javanese medakas were collected from estuary area in Bagan Lalang, Selangor, Malaysia (2°37’15.2”N 101°42’38.4”E). The fish were acclimatized from original salinity level where the fish were captured to final 0 ppt at 1 - 2 ppt/day. The fish were placed in 30 L holding tank with dechlorinated tap water and continuous aeration. The fish were also acclimatized and quarantined for 10 days prior to the experiment. The fish were fed twice daily with commercial brine shrimps and dry foods. Parasitic screening, together with bacterial isolation and identification from 30 randomly selected fish did not reveal any pathogenic organisms.

Bacterial Strains and Preparation

Stock culture of *A. hydrophila* (Saleema, 2015), *V. alginolyticus* (Nehlah et al., 2017) and *V. harveyi* (Ina-Salwany & Nurhidayu, 2015) from previous studies were used. The stock of *A. hydrophila* was cultured in *Aeromonas* medium base agar (AMBA) (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 30°C. Then, five colonies of the isolates were inoculated into 10 mL of tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and incubated in an orbital incubator at 0.75 × *g* for 24 h and at 30°C.

For *V. alginolyticus* and *V. harveyi*, the bacterial stocks were separately cultured on tryptic soy agar (TSA) (Merck) with 1.2% NaCl, and incubated for 24 h at 30°C. Then, five colonies of the isolates were inoculated into 10 mL of TSB with 1.2% NaCl, and incubated in an orbital incubator at 0.75 × *g* for 24 h and at 30°C.

Experimental Conditions for Fish Challenge Study

A total of 1080 Javanese medakas were used for determination of $LD_{50\text{-}240h}$ for IP and IMM routes for *A. hydrophila*, *V. alginolyticus* and *V. harveyi* (180 Javanese medakas for each IP and IMM route, for each bacterial species). During the challenge study, the fish were placed in 6 L aquariums. The static water system was used throughout the study period, while a portion of water was changed daily. Each treatment was conducted in triplicate, including the control group. Every replicate contained 60 fish (10 fish each for five bacterial concentrations and one negative control). The experiments were conducted for 240 h. The freshly dead fish in each treatment group was also collected for bacterial isolation and identification following bacterial challenge.

The mean ± standard deviation (SD) of the total length and weight of the fish prior to the experiments were 1.41 ± 0.23 cm and 0.109 ± 0.091 g, respectively. The measurements were obtained from 20 randomly sampled fish. The water quality parameters were monitored daily. The mean ± SD of water temperature (28.10 ± 0.10°C), pH (7.1 ± 0.7) and dissolved oxygen (5.49 ± 0.08 mg/L) were measured using a hand-held YSI meter (YSI, Yellow Springs, OH, USA), while nitrite (0.12 ± 0.07 mg/L) and ammonia (0.03 ± 0.01 mg/L) were determined using a spectrophotometer (HACH Company, Loveland, CO, USA).

Determination of $LD_{50\text{-}240h}$ in IP Route

The bacterial concentrations were calculated based on standard ten-fold serial dilutions and spread plating onto AMBA for *A. hydrophila*, and TSA with 1.2% NaCl for *V. alginolyticus* and *V. harveyi*.

With the exception of the control group, five different bacterial concentrations were used for each IP route challenge (Table 1). The bacterial concentrations ranged as follows: *A. hydrophila* from 2.5×10^5 CFU/mL to 2.5×10^9 CFU/mL, *V. alginolyticus* from 7.0×10^3 CFU/mL to 7.0×10^7 CFU/mL, and *V. harveyi* from 7.0×10^3 CFU/mL to 7.0×10^7 CFU/mL.

The fish were IP-challenged with bacteria according to previous protocols (Phelps et al., 2009; Patterson et al., 2012). Briefly, the fish were anesthetized using MS222 (Sigma-Aldrich, Kuala Lumpur, Malaysia) at the concentration of 0.168 mg/mL (Amal et al., 2018). Then, they were placed with the abdomen facing upwards and supported by a moistened foam bed to ensure that they remained in an upside-down position. A 31G (0.25 mm diameter and 8 mm length) syringe needle (Terumo, Somerset, NJ, USA) was used to administer 20 µL of the bacterial suspension into the peritoneal cavity of the fish. The needle was inserted into the midline of the abdomen, posterior to the pectoral fins. Only the tip of the needle was inserted into the abdomen of each fish to prevent damage to the internal organs. The $LD_{50\text{-}240h}$ was calculated by formulating the regression equations from the percentage of mortality of the fish *versus* the concentrations of the respective bacteria used (Zakari & Kubmarawa, 2016).

Determination of $LD_{50\text{-}240h}$ in IMM Route

The bacterial concentrations were calculated as mentioned above. With the exception of the control group, five different bacteria concentrations were used for each IMM route challenge (Table 1). The bacteria concentrations ranged as follows: *A. hydrophila* from 5.6×10^3 CFU/mL to 5.6×10^7 CFU/mL, *V. alginolyticus* from 3.5×10^4 CFU/mL to 3.5×10^8 CFU/mL, and *V. harveyi* from 5.2 CFU/mL to 5.2×10^4 CFU/mL.

For IMM route, prior to the procedures, a 6 L recovery aquarium containing de-chlorinated water was prepared. Ten fish were caught using a sterile fish net and were immersed in their respective bacterial suspension and was left for 2 min. The fish was then transferred into the recovery aquarium to wash off any excessive suspension before transferring them back into their respective post-treatment aquarium with another sterile fish net to avoid contamination. Prior to the challenge, the fish were anesthetized using MS222 as mentioned above. The $LD_{50\text{-}240h}$ was calculated as mentioned above.

Histopathological Assessments

The freshly dead Javanese medakas in each treatment were collected for histopathological assessment. Following fixation in 10% buffered formalin for 24 - 48 h, the fish samples were then processed in tissue processor (Leica TP 1020, Germany), embedded in paraffin, sectioned at 4 µm thick (Leica Jung Multicut 2045, Germany) and stained routinely with Harris haematoxylin and eosin (HE) for histological study. Complete sections of the fish in each group were examined to detect pathological changes. The photos were recorded and analyzed through microscope Nikon Eclipse 50i Japan and The Nikon NIS-Element D 3.2 Image Analyzer (Nikon Instruments Inc., USA).

RESULTS

Clinical Signs and Gross Lesions of Infected Javanese Medakas

The main clinical signs and gross lesions of Javanese medaka infected by *A. hydrophila*, *V. alginoluticus* and *V. harveyi* are presented in Figures 1 to 3. For *A. hydrophila*, the main observation of the IP and IMM challenged

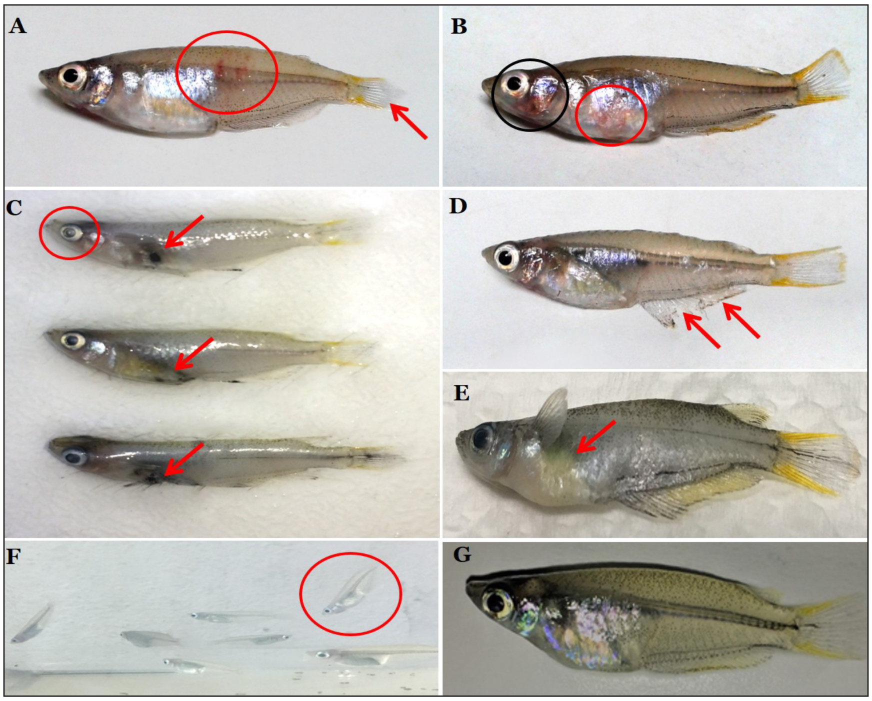

Figure 1. The main clinical signs and gross lesions of Javanese medakas infected by *Aeromonas hydrophila* following IP and IMM routes including A) inflammation of the dorsal part (red circle) and necrotic caudal fin (red arrow); B) inflammation of the opercular (black circle) and abdominal (red circle) area; C) cloudy eye (red circle) and necrosis of the injection site (red arrow) which only observed in the IP challenged fish; D) necrosis at the edge of anal fin; E) greenish and enlarged of gall bladder; F) occasionally erratic swimming patterns; and G) control fish

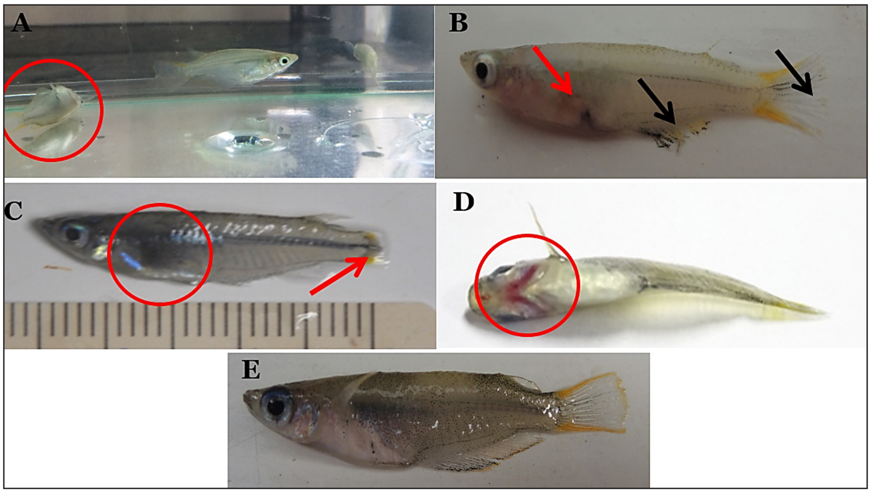

Figure 2. The main clinical signs and gross lesions of Javanese medakas infected by *Vibrio alginolyticus* following IP and IMM routes including A) abnormal swimming patterns (red circle); B) necrosis at injection site (red arrow) which only found in the IP challenged fish, and necrosis of caudal and anal fins (black arrow); C) darkening of the abdominal (red circle) and severe necrosis of caudal fin (red arrow); D) hemorrhage of opercular (red circle) and; E) control fish

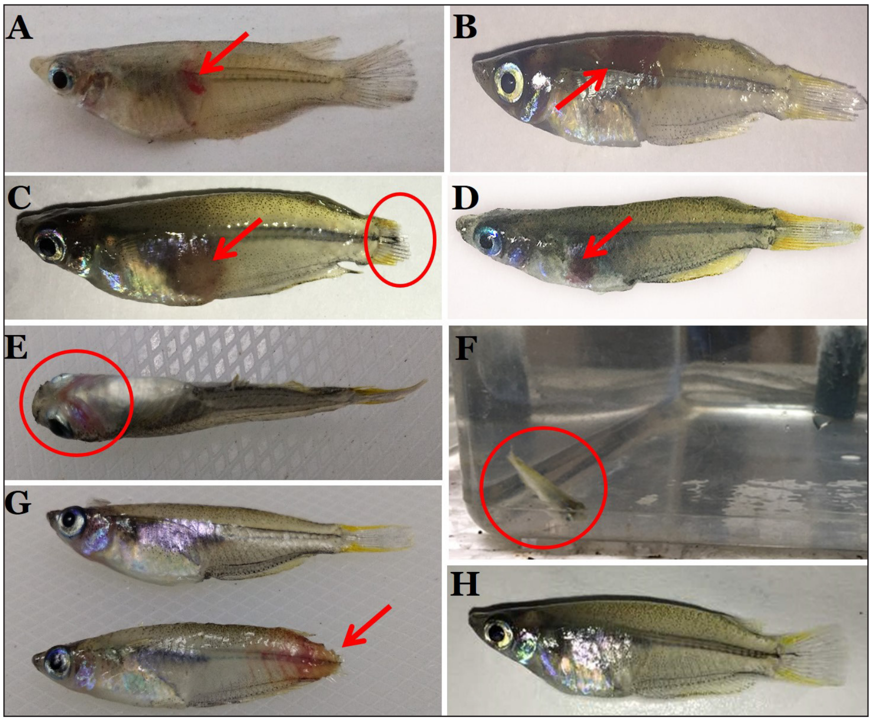

Figure 3. The main clinical signs and gross lesions of Javanese medakas infected by *Vibrio harveyi* following IP and IMM routes including A) hemorrhage of the body muscle (red arrow); B) hemorrhage of the dorsal body (red arrow); C) darkening of the abdomen (red arrow) and necrosis of caudal fin (red circle); D) hemorrhage of abdomen; E) inflammation of opercular area (red circle); F) isolated, less responsive and lethargic (red circle); G) severe necrosis of caudal fin (red arrow); and H) control fish

fish included occasionally erratic swimming patterns, inflammation of the dorsal, opercular and abdominal areas, necrotic caudal fin, and edge of anal fin, cloudy eye, enlarged gall bladder, and necrosis at the injection site for IP challenged fish only.

For *V. alginolyticus*, the main finding of the IP and IMM challenged fish included abnormal swimming patterns, necrosis of caudal and anal fins, darkening of the abdominal part, hemorrhage of opercula and necrosis at the injection site in the IP challenged fish only. The main abnormalities observed in IP and IMM infected Javanese medaka by *V. harveyi* generally showed similar observation following infection by *V. alginolyticus*, including isolated, less responsive and lethargic, hemorrhage of the body muscle and dorsal body part, darkening of the abdomen, necrosis of caudal fin, inflammation of the opercular area, and severe necrosis of caudal fin.

$LD_{50\text{-}240h}$ and Mortality Patterns Following IP and IMM Challenges

The $LD_{50\text{-}240h}$, mortality patterns and total mortality due to *A. hydrophila, V. alginolyticus* and *V. harveyi* in Javanese medakas following IP and IMM challenges up to 240 hpi are presented in Table 1. The $LD_{50\text{-}240h}$ of each bacteria in Javanese medakas were lower in IP route, compared with IMM route. For IP challenge, the lowest $LD_{50\text{-}240h}$ was observed for *V. harveyi* with 2.1×10^4 CFU/mL, while the highest observed for *A. hydrophila* with 2.5×10^7 CFU/mL, while for IMM challenge, the lowest $LD_{50\text{-}240h}$ was also observed for *V.*

Table 1
Mortality patterns, total mortality and $LD_{50\text{-}240h}$ of Aeromonas hydrophila*,* Vibrio alginolyticus *and* V. harveyi *in Javanese medakas following IP and IMM challenges up to 240 hpi*

Pathogen concentration (CFU/mL)	Hours post infection										Total mortality	% mortality
	24	48	72	96	120	144	168	192	216	240		
					Aeromonas hydrophila							
Intraperitoneal	$LD_{50\text{-}240h} = 2.5 \times 10^7$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0.0
2.5×10^5	6	2	1	0	0	0	0	2	1	0	12	16.0
2.5×10^6	4	1	0	2	1	1	0	0	1	3	13	43.3
2.5×10^7	7	1	1	1	0	0	1	1	1	2	15	50.0
2.5×10^8	5	2	0	2	0	2	0	2	2	1	16	53.3
2.5×10^9	11	3	0	0	0	2	0	2	1	0	19	63.3
Immersion	$LD_{50\text{-}240h} = 3.1 \times 10^8$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0
5.6×10^3	0	0	0	0	1	0	0	1	0	0	2	6.7
5.6×10^4	0	0	1	0	0	0	1	1	2	0	5	16.7
5.6×10^5	0	0	2	0	3	0	0	2	0	1	8	26.7
5.6×10^6	0	0	1	0	2	2	1	2	1	1	10	33.3
5.6×10^7	0	0	1	0	1	1	3	3	2	2	13	43.3

Table 1 *(continue)*

Pathogen concentration (CFU/mL)	Hours post infection										Total mortality	% mortality
	24	48	72	96	120	144	168	192	216	240		
Vibrio alginolyticus												
Intraperitoneal	$LD_{50\text{-}240h} = 6.3 \times 10^5$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0.0
7.0×10^3	2	3	1	0	0	0	0	0	0	0	6	20.0
7.0×10^4	5	3	1	0	0	0	0	0	0	0	9	30.0
7.0×10^5	8	8	2	0	0	0	0	0	0	0	18	60.0
7.0×10^6	12	8	1	0	0	0	0	0	0	0	21	70.0
7.0×10^7	10	10	1	1	0	0	0	0	0	0	22	73.3
Immersion	$LD_{50\text{-}240h} = 1.1 \times 10^9$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0.0
3.5×10^4	0	0	0	1	0	0	0	0	0	0	1	3.3
3.5×10^5	0	0	1	0	0	0	0	0	0	0	1	3.3
3.5×10^6	0	0	1	0	1	0	0	0	0	0	2	6.7
3.5×10^7	0	0	1	0	1	0	0	0	0	0	2	6.7
3.5×10^8	0	4	1	1	0	0	0	0	0	0	6	20.0
Vibrio harveyi												
Intraperitoneal	$LD_{50\text{-}240h} = 2.1 \times 10^4$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0
7.0×10^3	12	3	0	0	0	0	0	0	0	0	15	50.0
7.0×10^4	16	0	0	0	0	0	0	0	0	0	16	53.3
7.0×10^5	17	0	0	0	0	0	0	0	0	0	17	56.7
7.0×10^6	18	5	0	0	0	0	0	0	0	0	23	76.7
7.0×10^7	25	1	0	0	0	0	0	0	0	0	26	86.7
Immersion	$LD_{50\text{-}240h} = 6.6 \times 10^7$ CFU/mL											
Control	0	0	0	0	0	0	0	0	0	0	0	0
5.2	0	0	0	0	1	1	0	1	0	0	3	10.0
52.0	0	1	0	0	0	0	3	0	0	0	4	13.3
5.2×10^2	0	1	0	0	0	2	1	1	0	0	5	16.7
5.2×10^3	1	1	0	1	1	2	0	0	0	0	6	20.0
5.2×10^4	1	1	2	0	1	1	3	1	0	0	10	33.3

harveyi with 6.6×10^7 CFU/mL, while the highest was for *V. alginolyticus* with 1.1×10^9 CFU/mL.

Moreover, in IP route, most of the fish mortalities (88.9%) were observed at ≤ 120 hours post infection (hpi), while in IMM, most of the mortalities (50.6%) were witnessed beyond 120 hpi. No mortality was recorded for control group either in IP or IMM route for all of the bacterial treatments. *Aeromonas hydrophila*, *V. alginolyticus* and *V. harveyi* were successfully isolated from the freshly dead fish in their respective challenge group either in IP or IMM route.

Histopathological Assessments Following IP and IMM Challenges

Javanese medakas infected with *A. hydrophila* showed moderate meningeal congestion (Figure 4A). Mild glomerulitis was observed with different degrees of hyalinization in the glomeruli (Figure 4B). Formation of cytoplasmic vacuolation within the hepatocytes was observed (Figure 4C). Infiltration of inflammatory cells in between muscle bundle indicating moderate myositis (Figure 4D).

Javanese medakas infected with *V. alginolyticus* displayed moderate meningeal congestion (Figure 5A). However, in *V. harveyi* infection group, congestion of blood vessels in the cerebrum was observed as well, apart from the meningeal congestion (Figure 6A). The kidney of *V. alginolyticus* infection group demonstrated intratubular hemorrhage (Figure 5C). However, in *V. harveyi* infection group, discrete mild tubular necrosis and moderate atrophic glomeruli can be observed in IP and IMM routes respectively (Figure 6B, 6D). In *V. alginolyticus* infection group, mild hepatic necrosis with diffused pattern was observed (Figure 5B), while multifocal patterns were observed in *V. harveyi* infected group (Figure 6C). Finally, Javanese medakas infected with *V. alginolyticus* displayed increase in MMC formation (Figure 5D). The normal histology of Javanese medakas from negative control groups as presented in Figure 7. The clinical signs, gross lesions and histopathological changes of Javanese medakas following IP and IMM infection by *A. hydrophila*, *V. alginolyticus* and *V. harveyi* as summarized in Table 2.

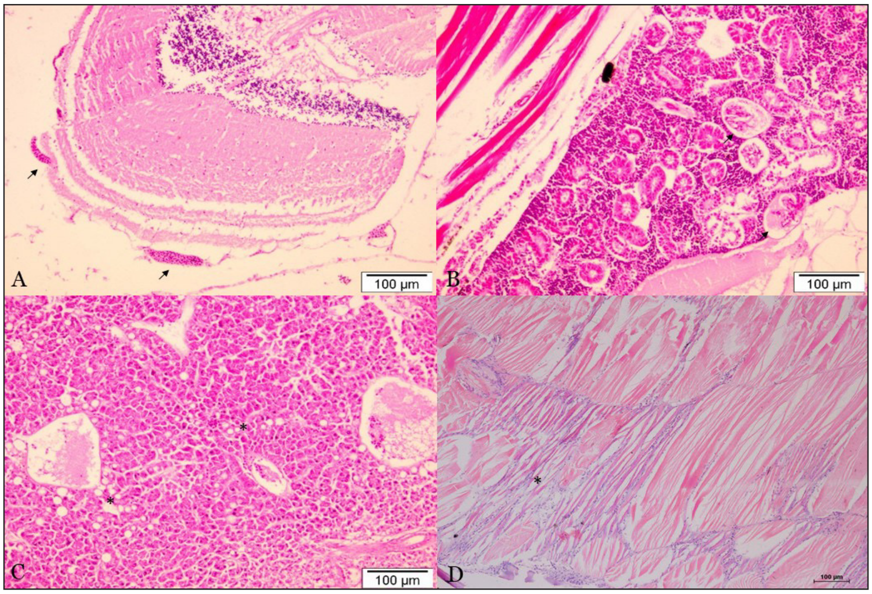

Figure 4. The main histopathological findings of Javanese medakas infected by *Aeromonas hydrophila* through IMM route (A, B, C) and IP route (D). A) Moderate meningeal congestion (arrows), brain, ×100, HE; B) Mild glomerulitis (arrow), kidney, ×100, HE; C) Mild hepatocytes cytoplasmic vacuolation (*), liver, ×100, HE; D) Moderate myositis (*), muscle, ×100, HE

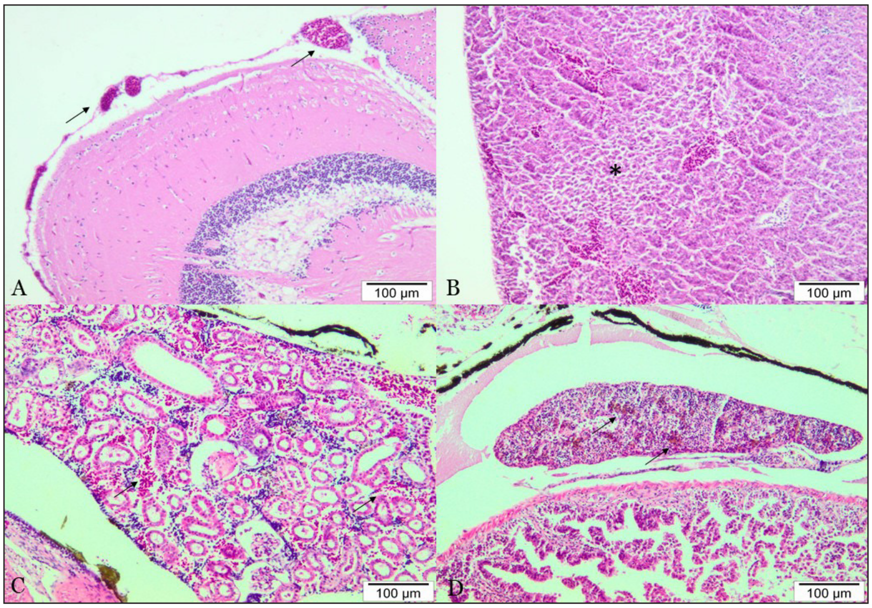

Figure 5. The main histopathological findings of Javanese medakas infected by *Vibrio alginolyticus* through IP route (A, B) and IMM route (C, D). A) Moderate meningeal congestion (arrows), brain, ×100, HE; B) Mild hepatic necrosis (*), liver, ×100, HE; C) Mild renal intratubular hemorrhage (arrows), kidney, ×100, HE; D) Hyper aggregation of splenic melano macrophage centre (MMC) (arrows), spleen, ×100, HE

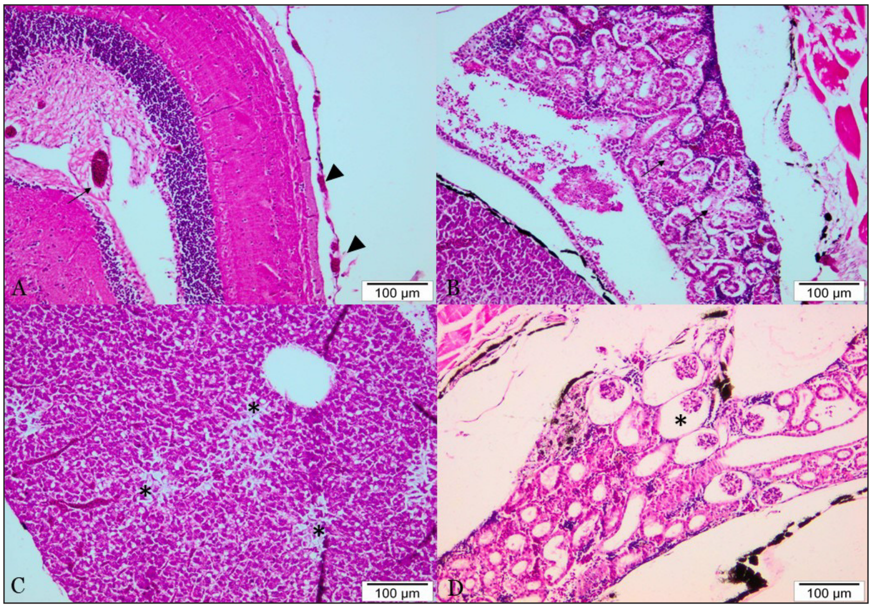

Figure 6. The main histopathological findings of Javanese medakas infected by *Vibrio harveyi* through IP route (A, B) and IMM route (C, D). A) Mild congestion of blood vessels in cerebrum (arrow) and meninges layer (arrow head), brain, ×100, HE; B) Mild tubular necrosis (arrows), liver, ×100, HE; C) Multifocal hepatic necrosis (*), ×100, HE; D) Moderate atrophic glomeruli (*), kidney, ×100, HE

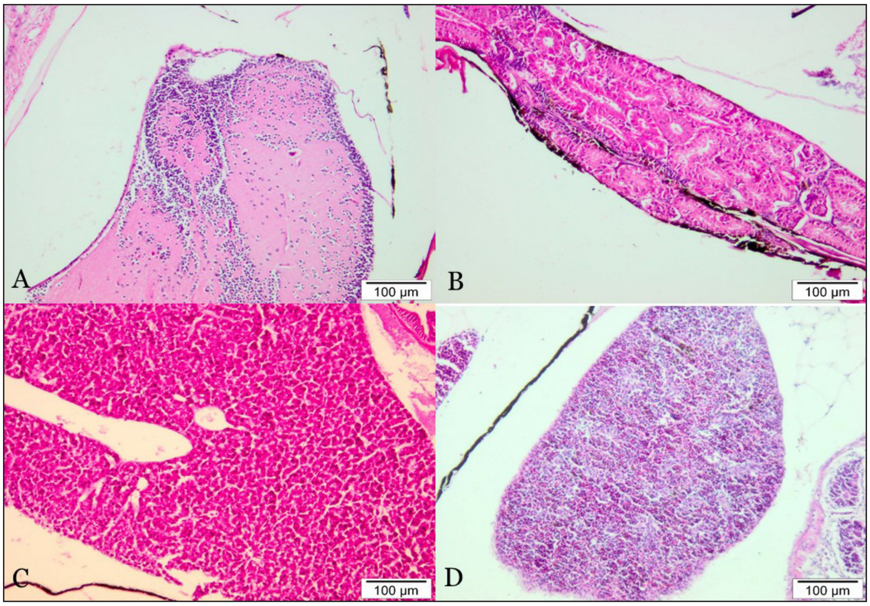

Figure 7. The normal histology of Javanese medakas from negative control groups. A) Brain, ×100, HE; B) Kidney, ×100, HE; C) Liver, ×100, HE; D) Spleen, ×100, HE

Table 2
Summary of clinical signs, gross lesions and histopathological changes of Javanese medakas following IP and IMM infections by Aeromonas hydrophila*,* V. alginolyticus *and* V. harveyi

Changes	Pathogen		
	Aeromonas hydrophila	*Vibrio alginolyticus*	*Vibrio harveyi*
Clinical signs	• Erratic swimming patterns	• Abnormal swimming patterns	• Isolated, less responsive and lethargic
Gross lesions	• Inflammation of the dorsal, opercular and abdominal areas • Necrotic of caudal fin and edge of anal fin • Cloudy eye • Greenish and enlarged gall bladder • Necrosis at the injection site for IP challenged fish	• Necrosis of caudal and anal fins • Darkening of the abdominal part • Hemorrhage of opercula • Necrosis at the injection site in the IP challenged fish	• Hemorrhage of the body muscle and dorsal body part • Darkening of the abdomen • Necrosis of caudal fin • Inflammation of the opercular area • Severe necrosis of caudal fin
Histo-patho-logical changes	• Moderate meningeal congestion • Mild glomerulitis was observed with different degrees of hyalinization in the glomeruli • Formation of cytoplasmic vacuolation within the hepatocytes • Infiltration of inflammatory cells in between muscle bundle indicating moderate myositis	• Moderate meningeal congestion • Intratubular hemorrhage of kidney • Mild hepatic necrosis with diffused pattern • Increase in MMC formation	• Congestion of blood vessels in the cerebrum • Meningeal congestion • Mild tubular necrosis and moderate atrophic glomeruli • Mild hepatic necrosis with multifocal patterns

DISCUSSION

Many animal species of interest have been selected as laboratory model organisms. Generally, these organisms were chosen as model animal due to their biological criteria, which are easy to handle and able to represent a bigger group of organisms (Ribas & Piferrer, 2014). Model organisms are usually known as the species of non-human used in the study of biology, to be understood their biological phenomena, thus, other raising models and theories can be applied towards other organisms, predominantly the more complex species compared with the original (Sabrina & Ankeny, 2013).

Medaka has been widely used as the test organism in ecology, genetics, toxicology and ecotoxicology (Kinoshita et al., 2009), but less utilized for fish-pathogen related study (Amal et al., 2018, 2019). In the present study, we discovered the suitability of Javanese medaka as potential model organism for *A. hydrophila*, *V. alginolyticus* and *V. harveyi* infections study. This study revealed that Javanese medaka is susceptible to all of the tested pathogens, causing mortalities and producing lesions typical of the diseases. The clinical signs, gross lesions and histopathological changes of Javanese medakas infected by each of the tested pathogens in this study were similarly observed in the real host of the pathogen and zebrafish (Dias et al., 2016; Laith & Najiah, 2013; Nurliyana et al., 2019c, d; Ransangan & Mustafa, 2009; Tendencia, 2002). This indicates the sensitivity of Javanese medaka to these pathogens and reproducibility of the infection in the fish, which are important criteria for a model organism (Dietrich et al., 2014).

Natural infection of *A. hydrophila* in catfish (*Clarias. gariepinus*) exhibited gross lesions such as inflammation of the dorsal fin base and body, and necrosis of the caudal fin, anal fin and body (Laith & Najiah, 2013; Saleema, 2015). In its natural host, manifestations of *V. alginolyticus* and *V. harveyi* infections in Asian seabass either as hemorrhagic patches, deep skin and fin ulceration, dark pigmentation, lack of appetite, presence of ascites in the body cavity, enlarged liver and spleen, swimming at the water surface and mortality (Ransangan & Mustafa, 2009; Sharma et al., 2013; Nurliyana et al., 2019c), were similarly observed in Javanese medakas in this study.

Infection of *A. hydrophila* in zebrafish was characterized by the distended visceral cavity and abdominal haemorrhages (Rodríguez et al., 2008). In this study, infection by *A. hydrophila* in Javanese medakas either by IP or IMM route involved muscular lesions, which is considered as classical *Aeromona*s infection (Roberts, 2012). It was believed that *A. hydrophila* used in this study was limited to inducing ulcerative skin lesions, with no hemorrhagic septicemia as in the virulent motile aeromonad septicemia strain (Dias et al., 2016). Experimental infections of *V. alginolyticus* and *V. harveyi* in zebrafish were never studied, however, *V. parahaemolyticus* and *V. anguillarium* infections in zebrafish demonstrated either hemorrhage of the abdominal cavity and surrounding the injection site (Liu et al., 2015; Paranjpye et al., 2013).

In this study, the $LD_{50\text{-}240h}$ of *A. hydrophila* in Javanese medaka was 2.5 × 10^7 CFU/mL and 3.1 × 10^8 CFU/mL in IP and IMM route, respectively, higher than observed in IP infected zebrafish at 4.0 × 10^5 CFU/mL (Rodríguez et al., 2008). However, in piracucu (*Arapaima gigas*), the $LD_{50\text{-}96h}$ of *A. hydrophila* was recorded at 1.8 × 10^8 CFU/mL following intradermal route of infection (Dias et al., 2016). The $LD_{50\text{-}240h}$ of *V. alginolyticus* and *V. harveyi* in Javanese medaka following IP and IMM challenges in this study were range from 2.1 × 10^4 CFU/mL to 6.3 × 10^5 CFU/mL, and 6.6 × 10^7 CFU/mL to 1.1 × 10^9 CFU/mL, while the previous study found that the LD_{50} of IP infected wild-type zebrafish with *V. parahaemolyticus* was 5.46 × 10^5 CFU/mL (Paranjpye et al., 2013). In cobia (*Rachycentron canadum*), the LD_{50} of *V. alginolyticus* was reported at 3.28 × 10^4 CFU/g fish body weight (Liu et al., 2004), while the LD_{50} of *V. harveyi* strain VHJR7 in Asian seabass was 1.4 × 10^4 CFU/g fish body weight (Ransangan et al., 2012). It is suggested that the varied LD_{50} obtained among the pathogens may explain that each pathogen tested have their own virulence factors, while different hosts also have different immunological reactions towards the pathogens.

The mean of $LD_{50\text{-}240h}$ for combined IP and IMM routes for each bacteria suggested that *V. harveyi* ($10^{5.5}$ CFU/mL) was the most pathogenic bacterium in this study, followed by *V. alginolyticus* (10^7 CFU/mL) and *A. hydrophila* ($10^{7.5}$ CFU/mL). However, in term of the route of infection, IP was considered more acute compared with chronic infection by IMM. This evidence based on the mortality patterns in which 88.9% of the fish mortalities were observed within ≤ 120 hpi in IP route, compared with 50.6% mortalities beyond 120 hpi in IMM route. This observation was similarly reported in zebrafish (Patterson et al., 2012), thus indicating the suitability of Javanese medaka as test organism for the bacterial pathogens.

Histopathological changes of experimentally infected striped catfish (*Pangasianodon hypophthalmus*) with *A. hydrophila* including necrotic hepatocytes, pyknosis, and vacuolation in the liver (Nahar et al., 2016), as similarly observed in this study. Moreover, the histopathological findings in Javanese medaka following *V. alginolyticus* and *V. harveyi* infections are generally similar. Histopathological examination of the Javanese medakas showed congestion, hemorrhage and necrosis in vital organs, especially the brain, liver and kidney. All of these findings were similarly observed in experimental and natural infections of *V. alginolyticus* in Asian seabass (Sharma et al., 2013) and natural infections of *V. harveyi* in Arabic surgeon (*Acanthurus sohal*) (Hashem & El-Barbary, 2013).

CONCLUSION

This study revealed that Javanese medaka was susceptible and sensitive towards IP and IMM infections by *A. hydrophila*, *V.*

alginolyticus and *V. harveyi*. The infections led to typical clinical signs, gross lesions, mortality and produced identical lesions of the diseases as observed in their natural hosts and zebrafish. This study highlights the potential utilization of Javanese medaka as another valuable *in vivo* model organism for bacterial disease studies in fish.

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REFERENCES

Amal, M. N. A., Ismail, A., Zamri-Saad, M., Ina-Salwany, M. Y., Shaqinah, N. N., Mastor, S. S., … Nurliyana, M. (2019). Study on *Streptococcus agalactiae* infection in Javanese medaka (*Oryzias javanicus* Bleeker, 1854) model. *Microbial Pathogenesis*, *131*(6), 47-52.

Amal, M. N. A., Zarif, S. T., Suhaiba, M. S., Aidil, M. R. M., Shaqinah, N. N., Zamri-Saad, M., & Ismail, A. (2018). The effect of fish gender on susceptibility to acute *Streptococcus agalactiae* infection in Javanese medaka *Oryzias javanicus*. *Microbial Pathogenesis*, *114*(1), 251-254.

Azila, A., Ramli, R., Ridzuan, M. S. M., Murni, M., Hashim, S., Sudirwan, F., … Amal, M. N. A. (2017). The presence of Vibrionaceae, *Betanodavirus* and *Iridovirus* in marine cage-cultured fish: Role of fish size, water physicochemical parameters and relationships among the pathogens. *Aquaculture Reports*, *7*(8), 57-65.

Aziz, F. Z. A., Zulkifli, S. Z., Mohamat-Yusof, F., Amal, M. N. A., & Ismail, A. (2017). Histological study on mercury-induced gonadal impairment in Javanese medaka (*Oryzias javanicus*). *Turkish Journal of Fisheries and Aquatic Sciences*, *17*(3), 621-627.

Bakar, N. A., Sata, N. S. A. M., Ramlan, N. F., Ibrahim, W. N. W., Zulkifli, S. Z., Abdullah, C. A. C., … Amal, M. N. A. (2017). Evaluation of the neurotoxic effects of chronic embryonic exposure with inorganic mercury on motor and anxiety-like responses in zebrafish (*Danio rerio*) larvae. *Neurotoxicology and Teratology*, *59*(1-2), 53-61.

Bleeker, P. (1854). Ichthyologische waarnemingen, gedaan op verschillende reizen in de Residentie Banten [Ichthyological observations made on different journeys in the Residence Banten]. *Natuurkundig Tijdschrift voor Nederlandsch Indië*, *7*, 309-326.

Dias, M. K. R., Sampaio, L. S., Proietti-Junior, A. A., Yoshioka, E. T. O., Rodrigues, D. P., Rodriguez, A. F. F., … Tavares-Dias, M. (2016). Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. *Veterinary Microbiology*, *188*(5), 12-15.

Dietrich, M. R., Ankeny, R. A., & Chen, P. M. (2014). Publication trends in model organism research. *Genetics*, *198*(3), 787-794.

Ferdaus, M. Y., Sarah-Nabila, A. G., Syaizwan, Z. Z., Amal, M. N. A., Wan-Norhamidah, W. I., Yusof, S., & Ismail, A. (2018). Acute toxicity test of copper pyrithione on Javanese medaka and the behavioural stress symptoms. *Marine Pollution Bulletin*, *127*(2), 150-153.

Haenen, O. L. M., Fouz, R. B., Amaro, G. C., Isern, M. M., Mikkelsen, H., Zrnčić, S., … Dalsgaard, I. (2014). Vibriosis in aquaculture. *Bulletin of the European Association of Fish Pathologists*, *34*(4), 138-147.

Hashem, M., & El-Barbary, M. (2013). *Vibrio harveyi* infection in Arabian surgeon fish (*Acanthurus sohal*) of Red Sea at Hurghada, Egypt. *Egyptian Journal of Aquatic Research*, *39*(3), 199-203.

Hossain, M. J., Sun, D., McGarey, D. J., Wrenn, S., Alexander, L. M., Martino, M. E., … Liles, M. R. (2014). An Asian origin of virulent *Aeromonas hydrophila* responsible for disease epidemics in United States-farmed catfish. *mBio*, *5*(3), e00848-14.

Imai, S., Koyama, J., & Fujii, K. (2007). Effects of estrone on full life cycle of Java medaka (*Oryzias javanicus*), a new marine test fish. *Environmental Toxicology and Chemistry*, *26*(4), 726-731.

Ina-Salwany, M. D., & Nurhidayu, A. S. (2015). A prospect of using *Vibrio harveyi* isolated from the intestine of healthy farmed tiger grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775) as probiotics. *CIBTech Journal of Biotechnology*, *4*(3), 25-35.

Ina-Salwany, M. Y., Al-saari, N., Mohamad, A., Fathin-Amirah, M., Mohd, A., Amal, M. N. A., … Zamri-Saad, M. (2018). Vibriosis in fish: A review on disease development and prevention. *Journal of Aquatic Animal Health*, *31*(1), 3-22.

Inoue, K., & Takei, Y. (2002). Diverse adaptability in *Oryzias* species to high environmental salinity. *Zoological Science*, *19*(7), 727-734.

Ismail, A., & Yusof, S. (2011). Effect of mercury and cadmium on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Marine Pollution Bulletin*, *63*(5-12), 347-349.

Ismail, M. S., Siti-Zahrah, A., Syafiq, M. R. M., Amal, M. N.A., Firdaus-Nawi, M., & Zamri-Saad, M. (2016). Feed-based vaccination regime against streptococcosis in red tilapia, *Oreochromis niloticus × Oreochromis mossambicus*. *BMC Veterinary Research*, *12*(1), 194.

Ismail, M. S., Syafiq, M. R., Siti-Zahrah, A., Fahmi, S., Shahidan, H., Hanan, Y., … Zamri-Saad, M. (2017). *Fish and Shellfish Immunology*, *60*(1), 21-24.

Kinoshita, M., Murata, K., Naruse, K., & Tanaka, M. (2009). *Medaka: Biology, management and experimental protocols*. Hoboken, USA: Wiley-Blackwell.

Laith, A. R., & Najiah, M. (2013). *Aeromonas hydrophila*: Antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). *Journal of Aquaculture Research and Development*, *5*(2), 1-7.

Liu, P. C., Lin, J. Y., Hsiao, P. T., & Lee, K. K. (2004). Isolation and characterization of pathogenic *Vibrio alginolyticus* from diseased cobia *Rachycentron canadum*. *Journal of Basic Microbiology*, *44*(1), 23-28.

Liu, X., Wu, H., Liu, Q., Wang, Q., Xiao, J., & Zhang, Y. (2015). Skin-injured zebrafish, *Danio rerio*, are more susceptible to *Vibrio anguillarum* infection. *Journal of the World Aquaculture Society*, *46*(3), 301-310.

Magtoon, W., & Termvidchakorn, A. (2009). A revised taxonomic account of ricefish *Oryzias* (Beloniformes; Adrianichthyidae), in Thailand, Indonesia and Japan. *Natural History Journal of Chulalongkorn University*, *9*(1), 35-68.

Nahar, S., Rahman, M. M., Ahmed, G. U., & Faruk, M. A. R. (2016). Isolation, identification, and characterization of *Aeromonas hydrophila* from juvenile farmed pangasius (*Pangasianodon hypophthalmus*). *International Journal of Fisheries and Aquatic Studies*, *4*(4), 52-60.

Naruse, K., Minoru, T., & Hiroyuki, T. (2011). *Medaka: A model for organogenesis, human disease, and evolution*. Tokyo, Japan: Springer.

Neely, M. N., Pfeifer, J. D., & Caparon M. (2002). *Streptococcus*-zebrafish model of bacterial pathogenesis. *Infection and Immunity*, *70*(7), 3904-3914.

Nehlah, R., Firdaus-Nawi, M., Nik Haiha, N. Y., Karim, M., Zamri-Saad, M., & Ina-Salwany M. Y. (2017). Recombinant vaccine protects juvenile hybrid grouper, *Epinephelus fuscoguttatus* ×

Epinephelus lanceolatus, against infection by *Vibrio alginolyticus*. *Aquaculture International*, *25*(6), 2047-2059.

Norhariani, M. N., Siti-Hajar, M. Y., Hassan, M. D., Amal, M. N. A., & Nurliyana, M. (2019). Costs of management practices of Asian seabass (*Lates calcarifer* Bloch, 1790) cage culture in Malaysia using stochastic model that include uncertainty in mortality. *Aquaculture*, *510*, 347-352. doi: 10.1016/j.aquaculture.2019.04.042

Nurliyana, M., Amal, M. N. A., Zamri-Saad, M., & Ina-Salwany, M. Y. (2019a). Possible transmission routes of *Vibrio* spp. in tropical cage-cultured marine fishes. *Letters in Applied Microbiology*, *68*(6), 485-496.

Nurliyana, M., Amal, M. N. A., Zamri-Saad, M., Ina-Salwany, M. Y., Nor-Amalina, Z., Muskhazli, M., & Shaqinah, N. N. (2019b). Virulence-associated genes and antibiotic resistance patterns of *Vibrio* spp. isolated from cultured marine fishes in Malaysia. *BMC Veterinary Research*, *2019*(15), 176.

Nurliyana, M., Fauzul-Aidil, M. R., Amal, M. N. A., Zamri-Saad, M., Ina-Salwany, M. Y., Nor-Amalina, Z., & Shaqinah, N. N. (2019c). Natural concurrent infection of *Vibrio harveyi* and *V. alginolyticus* in cultured hybrid groupers in Malaysia. *Journal of Aquatic Animal Health*, *31*(1), 88-96.

Nurliyana, M., Muskhazli, M., Amal, M. N. A., Zamri-Saad, M., Ina-Salwany, M. D., & Nurhidayu, A. (2019d). Environmental factors associated with the presence of Vibrionaceae in tropical cage-cultured marine fishes. *Journal of Aquatic Animal Health*, *31*(2), 154-167. doi: 10.1002/aah.10062.

Paranjpye, R. N, Myers, M. S., Yount, E. C., & Thompson, J. L. (2013). Zebrafish as a model for *Vibrio parahaemolyticus* virulence. *Microbiology*, *159*(12), 2605-2615.

Patterson, H., Saralahti, A., Parikka, M., Dramsi, S., Trieu-Cuot, P., Poyart, C., … Rämet, M. (2012). Adult zebrafish model of bacterial meningitis in *Streptococcus agalactiae* infection. *Developmental and Comparative Immunology*, *38*(3), 447-455.

Phelps, H. A., Runft, D. L., & Neely, M. N. (2009). Adult zebrafish model of streptococcal infection. *Current Protocols in Microbiology*, *13*(5), 9D-1.

Ransangan, J., & Mustafa, S. (2009). Identification of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*) by use of 16S ribosomal DNA sequencing. *Journal of Aquatic Animal Health*, *21*(3), 150-155.

Ransangan, J., Lal, T. M., & Al-Harbi, A. H. (2012). Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*). *Malaysian Journal of Microbiology*, *8*(2), 104-115.

Ribas, L., & Piferrer, F. (2014). The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture*, *6*(4), 209-240.

Roberts, R. J. (2012). *The bacteriology of teleosts*. Oxford, United Kingdom: Wiley-Blackwell.

Rodríguez, I., Novoa, B., & Figueras A. (2008). Immune response of zebrafish (*Danio rerio*) against a newly isolated bacterial pathogen *Aeromonas hydrophila*. *Fish and Shellfish Immunology*, *25*(3), 239-249.

Sabrina, L., & Ankeny, R. A. (2013). What makes a model organism?. *Endeavour*, *37*(4), 209-212.

Saleema, M. (2015). *Molecular characterization of* Aeromonas hydrophila *and development of recombinant cells vaccine expressing outer membrane proteins against its in African catfish (*Clarias gariepinus *Burchell)* (Unpublished Master thesis), Universiti Putra Malaysia, Malaysia.

Salleh, A. F. M., Amal, M. N. A., Shaqinah, N. N., Syaizwan, Z. Z., Ferdaus M. Y., Ibrahim, W. N. W., & Ismail, A. (2017). Water pH effects on survival, reproductive performances and ultrastructure of gonads, gills and skins of Javanese medaka (*Oryzias javanicus*). *Turkish Journal of Veterinary and Animal Sciences*, *41*(4), 471-481.

Schmidt, J. G., Korbut, R., Ohtani, M., & Jørgensen, L. V. G. (2017). Zebrafish (*Danio rerio*) as a model to visualize infection dynamics of *Vibrio anguillarum* following intraperitoneal injection and bath exposure. *Fish and Shellfish Immunology*, *67*(8), 692-697.

Sharma, S. R. K., Rathore, G., Verma, D. K., Sadhu, N., & Philipose, K. K. (2013). *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquaculture Research*, *44*(1), 86-92.

Tendencia, E. A. (2002). *Vibrio harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in the Philippines. *Aquaculture Research*, *33*(6), 455-458.

Termvidchakorn, A., & Magtoon, W. (2008). Development and identification of the ricefish *Oryzias* in Thailand. *ScienceAsia*, *34*(4), 416-423.

Toranzo, A. E., Magariños, B., & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, *246*(1-4), 37-61.

Yusof, S., Ismail, A., & Alias, M. S. (2014). Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Marine Pollution Bulletin*, *85*(2), 494-498.

Yusof, S., Ismail, A., Koito, T., Kinoshita, M., & Inoue, K. (2012). Occurrence of two closely related ricefishes, Javanese medaka (*Oryzias javanicus*) and Indian medaka (*O. dancena*) at sites with different salinity in Peninsular Malaysia. *Environmental Biology of Fishes*, *93*(1), 43-49.

Zakari, A., & Kubmarawa, D. (2016). Acute toxicity (LD_{50}) studies using Swiss albino mice and brine shrimp lethality (LC_{50} and LC_{90}) determination of the ethanol extract of stem bark of *Echinaceae angustifolia* DC. *Natural Products Chemistry and Research*, *4*(6), 1-6.

Zhu, Z. M., Dong, C. F., Weng, S. P., & He, J. G. (2017). The high prevalence of pathogenic *Vibrio harveyi* with multiple antibiotic resistance in scale drop and muscle necrosis disease of the hybrid grouper, *Epinephelus fuscoguttatus* (♀) × *E. lanceolatus* (♂), in China. *Journal of Fish Diseases*, *41*(4), 589-601.

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Morphometry and Natural Diets of *Distichodus engycephalus* from Middle Basin of Ogun River, Southwest Nigeria

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ABSTRACT

Morphometry, natural diets, length-weight parameters and condition factor of *Distichodus engycephalus* in middle basin of River Ogun, Southwest Nigeria were investigated. One hundred and fourteen fish were sampled; consisting of eighty-seven males and twenty-seven females. Stomach contents were analyzed, morphometric and meristic characters were also measured. The analyses of data were based on sexes and combined population using descriptive, regression and correlation statistics. Student t-test was employed to detect significant difference (α=0.05) between the mean characters of the sexes. Results showed that *D. engycephalus* is an herbivore; feeding mostly on *Oscillatoria* (45.6%), *Microspora* (43.9%) and *Protococcus* (43.9) by occurrence method. Numerically, the diets composed of *Oscillatoria* (40.0%), *Phormidium* (18.3%) and *Microcystis* (10.3%). The mean total length, weight and condition factor for both sexes were 24.9±0.64 cm, 204.6±16.02 g and 1.09±0.01, respectively. Student t-test statistics revealed that means of the investigated characters were significantly higher ($p<0.05$) in female fish except depth of caudal peduncle (DCP) and number of pelvic rays (PVR). However, no significant difference ($p>0.05$) was detected in the condition factor between the sexes. Observed sex ratio for male: female fish was 1:0.31. The 'b' values of length-weight relationship analysis were 2.90 for male, 2.97 (female) and 0.92 (both sexes) which were not significantly different ($p>0.05$) from 3; indicating isometric growth pattern. Conclusively, *D. engycephalus* in the middle basin of Ogun River is herbivorous and grows isometrically.

Keywords: Characters, combined sexes, growth, herbivorous, isometry, total length

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INTRODUCTION

The decline in inland fish production can be attributed to over-fishing, climate change, lack of management of water bodies and anthropogenic effects such as pollution. To meet the fish demand of the human population, there is need to carry out research on the diets of various wild fish species in order to increase the culturable species through aquaculture. The knowledge of natural diets of fish is important in understanding the growth of the fish in the natural environment and aquaculture. Food studies of fish provides insight into trophic relationship and interactions among species in the aquatic ecosystem. Fish diets influence its growth, well-being, fecundity and migration (Adeyemi et al., 2009). In ecological research, knowledge of diet compositions is essential as it reveals the source of the animal's food on which it depends for growth and development (Ahlbeck et al., 2012).

One of the most commonly used analyses of fisheries data is length-weight relationship (Mendes et al., 2004). Length-weight relationship is often used to estimate weight from length (Sinovcic et al., 2004). Like any other morphometric characters, it can be used for the differentiation of taxonomic unit and the relationship changes with various developmental stages in life (Thomas et al., 2003). It is important in assessing the relative well-being of a fish population (Bolger & Connoly, 1989). Moutopoulos and Stergiou (2002) noted that length-weight relationship was important in estimating population size of a fish stock and growth pattern in fisheries management for rational exploitation and comparative growth studies. It can also be used to determine possible differences between separate unit stocks of the same species (King, 2007). Also, Kulbick et al. (2005) stressed the importance of the length-weight relationship in modelling aquatic ecosystem.

Distichodus engycephalus was observed to be one of the commercially important fish species, especially during the dry season, in Ogun River. *Distichodus engycephalus* is cherished by consumers and has good taste. *Distichodus* is genus in the family Distichodontidae and it is indigenous to tropical Africa (Arawomo, 1982). Among others, Teugels et al. (1992) reported *Distichodus* species as major exploitable fish species and widely distributed in Nigeria, Niger, Sudan, Volta, Chad and Nile basins. Three species, *Distichodus brevipinnis, D. engycephalus* and *D. rostratus*, are common in Nigerian inland waters. Few literature on the length-weight relationship, condition factor and its biology have been published by Berte et al. (2008) and Shinkafi et al. (2013). However, there is paucity of information on its natural diets and morphometry and no study has been published on this fish species in Ogun River.

MATERIALS AND METHODS

Description of Study Area

Ogun River has coordinates of 3º 28′ E and 8º 41′ N from its source in Oyo State to 3º 25′ E and 6º 35′ N in Lagos where it enters the Lagos Lagoon (Ayoade et al., 2004) and a total surface area of 22.4 km^2 (Oketola et al., 2006) as shown in Figure 1. Two seasons are experienced along the drainage basin of Ogun River, the wet season (April - October) and dry season (November - March). Rocky drainage basin is the major characteristic of the middle basin of Ogun River which is very obvious during the dry season when the water level is low.

Collection of Fish Samples

Samples of *D. engycephalus* were collected every month from December 2015 to May 2016 from artisanal fishermen operating in the middle basin of Ogun River using unselective fishing gears such as cast nets, traps and set gillnets of different mesh sizes. The fish samples were transported immediately to the laboratory for analysis in boxes containing ice cubes.

Laboratory Procedures

In the laboratory, meristic and morphometric measurements were taken and recorded. Individual fish was weighed to the nearest 0.1g using top loading scale (Camry, model: EK5350). The morphometric characters measured were standard length (SL), forked length (FL), total length (TL) and body depth (BD) to the nearest 0.1 cm with the use of a measuring board. Others measurements were eye diameter (EYD), body width (BWD), snout length (SNL), head length (HLT), length of dorsal fin base (LBD), length of anal fin base (LAB), length of base of adipose fin (BAD), length of pelvic fin (LPVF) and length of pectoral fin (LPF) using digital venier calliper to the nearest 0.1 mm. Meristic characters investigated were total number of dorsal spines (DOS),

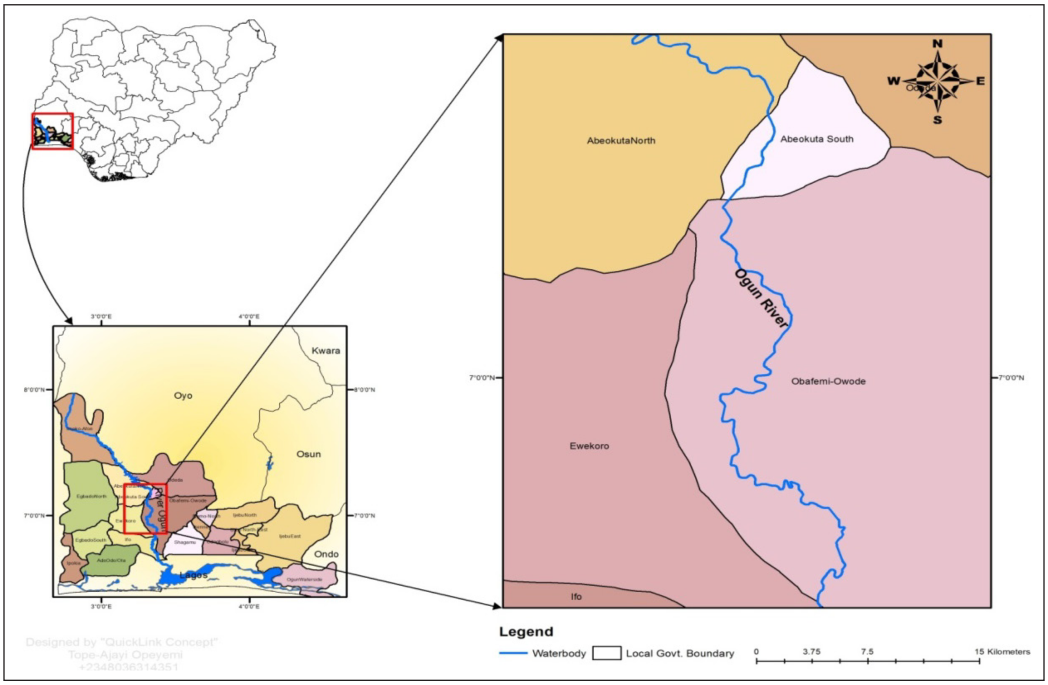

Figure 1. Map of Ogun River, Southwest Nigeria

dorsal rays (DRA), pelvic spines (PEVS), pelvic rays (PVR), pectoral spines (PECS), pectoral rays (PRA), anal spines (ANS) and anal rays (ARA). Individual fish was dissected to examine the sex.

Stomach Content Analysis

The degree of fullness were examined and reported as one-quarter (1/4), half (1/2), three-quarter (3/4) and one (1) for one-quarter, half, three-quarter and full stomach respectively. Each stomach was preserved with 4% neutral formalin separately in a labelled specimen bottle. Stomach wall was cut open longitudinally and contents were washed into the petri dish. The contents were analysed using frequency of occurrence and numerical methods according to Chipps and Garvey (2002).

Statistical Analyses

Descriptive and inferential statistics were used for the analysis. Graphs and tables were used to depict the results of the stomach contents. Scatter diagrams was employed to examine the length-weight relationship. Detected outliers were removed according to Froese (2006). Length-weight relationship parameters (a and b) were estimated by linear regression using logarithmic equation:

$$\log W = \log a + b \log L \quad \text{(Sparre \& Venema, 1992; Zar, 1984)} \qquad [1]$$

where W= weight (g), L = total length (cm), a = y-intercept (constant), b = slope of the graph. The length-weight relationship data were analyzed for male, female and both sexes. The b-values for male, female and combined sexes were tested if they were significantly different from 3 which is the isometric growth (Sokal & Rohlf, 1998). A one-way analysis of covariance (ANCOVA) was conducted to compare the regression slopes of the sexes whilst controlling for the effect of total length. The logarithm transformations of weight and total length were used in this wise. Levene's test and normality checks were carried out and the assumptions met. The condition factors (K) for male, female and combined sexes were calculated using the formula:

$$K = 100 \times [W/(L^3)] \qquad [2]$$

according to Bagenal and Tesch (1978), where K = condition factor, W = weight (g), L = total length (cm). Student t-test was used to test the null hypothesis (H_o) that the means of the investigated parameters of male and female fish are homogeneous ($\alpha = 0.05$). Correlation statistics was used to reveal the relationship among the investigated morphometric characters.

RESULTS AND DISCUSSION

In the study, a total of one hundred and fourteen (114) specimens were sampled consisting of eighty-seven males and twenty-seven females. The degree of stomach fullness ranged from 9% (empty) to 39% (full stomach) as shown in Figure 2. *Oscillatoria* (45.6%), *Protococcus* (43.9%), *Microspora* (43.9%) and *Nitzschia* (30.7%) were dominant natural diets by occurrence while the least comprised *Chroccocus*, *Asterocystis*, *Oocystis*, *Pinnularia*, *Docidium*, *Hydrodictyon*, *Scenedesmus*, *Coelosphaerium* with one percent each. This method depicts how often a particular food item was consumed but provides no information on the relative importance of the food to the overall diet. Numerical analysis showed that main diets were *Oscillatoria* (39.0%), *Phormidium* (18.3%) and *Microcystis* (10.3%) as depicted in Table 1. Figure 3 shows the categories of natural diets consumed by *D. engycephalus* in the middle basin of Ogun River, Nigeria. The main food category by occurrence was Chlorophyceae (47%), followed by Baccillariophyceae (18%) and Myxophyceae (13%) while numerical method revealed Myxophyceae (69%), Chlorophyceae (15%) and Bacillariophyceae (9%) as most preferred diets (Figure 4). Diets analysis showed that *D. engycephalus* in Ogun River is herbivorous; occupying low trophic level converting low energy substances to higher ones in the food chain. This observation supported Arawomo (1982) who reported that *D. engycephalus*, *D. brevipinnis* and *D. rostratus*, which were similar species in the same genus, in Kainji Lake were herbivores. Ahlbeck et al. (2012) stated that diet compositions revealed prey-predator relationships and potential competitors which contributed to the understanding of the population dynamics and functioning of ecosystem structure. Stomach contents in fishes reveal the role of the species in the integration and interaction of ecological components in the aquatic system which, among others, enhance ecosystem based management. However, location of sample, time of sampling in the year, characteristics of the habitat are factors that can influence the diet composition.

Total length of the specimens (both sexes) ranged between 12.3 and 40.3 cm with a mean of 24.9±0.64 cm and mean weight (204.6±16.02 g) varied between 26.0 and 791.0 g (Table 2). It was observed that total length and weight of female species of *D. engycephalus* in Ogun River were significantly higher ($p<0.05$) than that of male fish. The maximum total length observed in this study was higher than

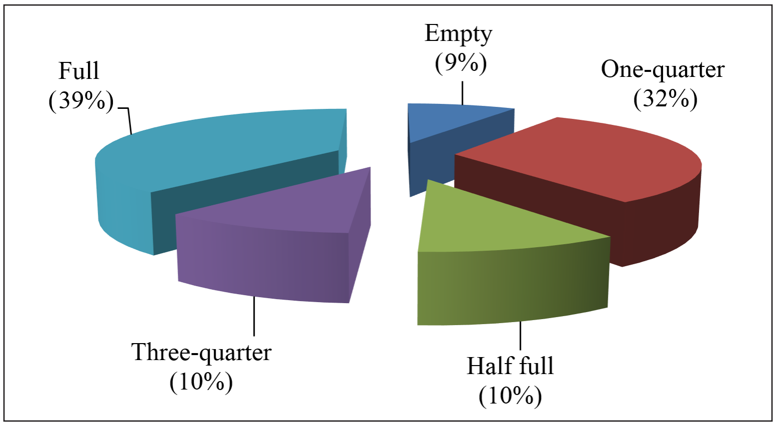

Figure 2. Stomach fullness of *Distichodus engycephalus* from middle basin of Ogun River, Southwest Nigeria

Table 1
Stomach content of Distichodus engycephalus *from middle basin of Ogun River, Southwest Nigeria*

Organism			Percentage	
	Occurrence method	Numeric method	Occurrence	Numeric
Bacillariophyceae	**11**	**27952**	**17.74**	**8.53**
Campylodiscus	6	10	5.263	0.003
Cyclotella	11	86	9.649	0.026
Cymbella	20	20	17.544	0.006
Diatoma	13	13	11.404	0.004
Gomphonema	12	12	10.526	0.004
Gyrosigma	5	2598	4.386	0.793
Navicula	4	4	3.509	0.001
Nitzschia	35	6035	30.702	1.842
Pinnularia	1	1	0.877	0.000
Synedra	15	22	13.158	0.007
Tabellaria	23	19151	20.175	5.846
Chlorophyceae	**29**	**50883**	**46.77**	**15.53**
Ankistrodesmus	3	3	2.632	0.001
Botryoccoccus	3	18	2.632	0.005
Bulbochaeta	4	4	3.509	0.001
Chaetophora	14	425	12.281	0.130
Characium	2	2	1.754	0.001
Chlorella	2	2	1.754	0.001
Cladophora	3	17	2.632	0.005
Closterium	15	161	13.158	0.049
Clucigenia	2	201	1.754	0.061
Coelastrum	5	5	4.386	0.002
Docidium	1	1	0.877	0.0003
Enteromorpha	6	79	5.263	0.024
Genicularia	23	23	20.175	0.007
Gonatozygon	16	3942	14.035	1.203
Hydrodictyon	1	1	0.877	0.000
Kirchneriella	5	380	4.386	0.116
Micrasteria	2	25	1.754	0.008
Microspora	50	14322	43.860	4.372
Mougeotia	2	1704	1.754	0.520
Oocystis	1	2	0.877	0.001
Protococcus	50	24660	43.860	7.528
Scenedesmus	1	1	0.877	0.000
Spirogyra	32	1132	28.070	0.346
Staurastrum	2	2	1.754	0.001
Stigeoclonium	3	11	2.632	0.003
Tetraspora	11	11	9.649	0.003

Table 1 *(continue)*

Organism			Percentage	
	Occurrence method	Numeric method	Occurrence	Numeric
Ulothrix	2	2	1.754	0.001
Volvox	2	2	1.754	0.001
Zygnema	4	3745	3.509	1.143
Cyanophyceae	**4**	**14943**	**6.45**	**4.56**
Calothrix	2	2	1.754	0.001
Coelosphaerium	1	1	0.877	0.000
Nostoc	2	2	1.412	0.001
Spirulina	11	14938	9.649	4.560
Insecta	**1**	**6**	**1.61**	**0.18**
Insects	6	6	5.263	0.002
Myxophyceae	**8**	**227216**	**12.9**	**69.36**
Anabaena	19	1030	16.667	0.314
Aphanizomenon	2	6	1.754	0.002
Chroococus	1	51	0.877	0.016
Glococapsa	22	4449	19.298	1.358
Mycrocystis	31	33933	27.193	10.358
Oscillatoria	52	127724	45.614	38.988
Phormidium	33	59992	28.947	18.313
Tolypotrix	6	31	5.263	0.009
Rodophycea	**4**	**5063**	**6.45**	**1.55**
Asterocystis	1	20	0.877	0.006
Batrachospermum	4	5028	3.509	1.535
Lamenea	3	8	2.632	0.002
Phorphyridium	3	7	2.632	0.002
Xanthophycae	**2**	**17**	**3.22**	**0.005**
Ophiocytium	3	3	2.632	0.001
Vaucheria	2	14	1.754	0.004
Nematoda	**1**	**184**	**1.61**	**0.06**
Nematode worm	22	184	19.298	0.056
Others	**2**	**1332**	**3.22**	**0.41**
Plant parts	33	1283	28.947	0.392
Fish Scales	16	49	14.035	0.015

those reported for *D. engycephalus* in Anambra River, Nigeria (Nwani & Ude, 2005) and Yapei Stretch of White Volta, Ghana (Abobi, 2015). However, Shinkafi et al. (2013) and Abowei (2010) reported maximum total length of 46.2 cm and 54.0 cm for *D. rostratus* in River Rima and lower Nun River, Nigeria respectively. Mean weight of 218.3 g, 332.0 g and 227.0 g were documented for male *D. engycephalus*, *D. brevipinnis and D. rostratus* and 213.7g, 218.3 g and 23.0 g for female respectively

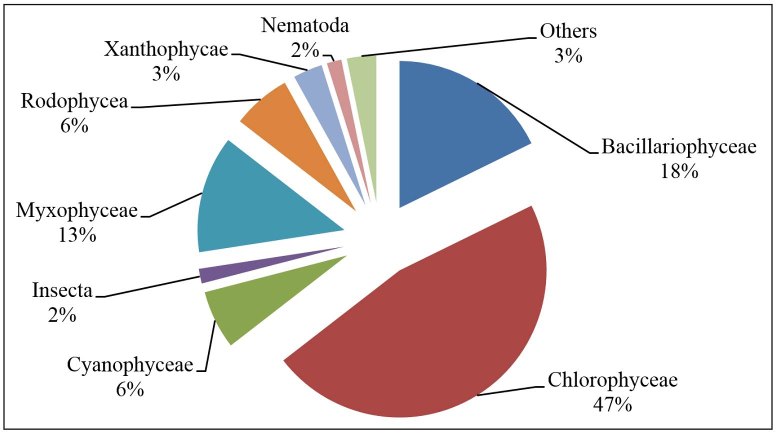

Figure 3. Categories of stomach content (occurrence method) of *Distichodus engycephalus* from middle basin of Ogun River, Southwest Nigeria

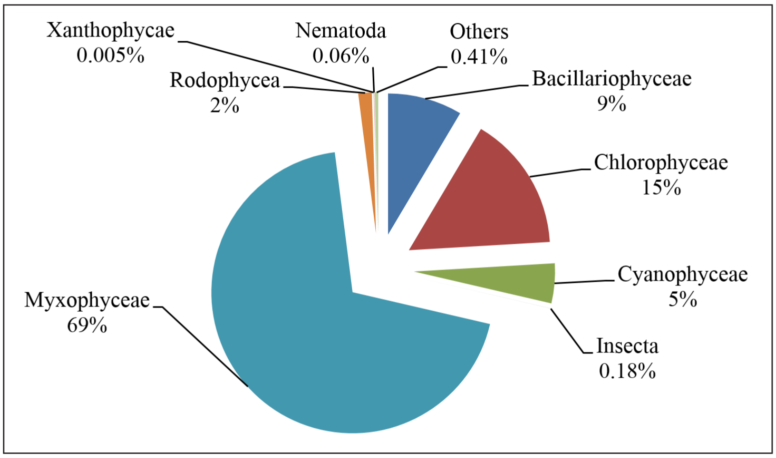

Figure 4. Categories of stomach content (numerical method) of *Distichodus engycephalus* from middle basin of Ogun River, Southwest Nigeria

Table 2
Morphometry of Distichodus engycephalus *from middle basin of Ogun River, Southwest Nigeria*

	Male			Female			Both sexes		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
WT	26.0	722.0	143.8±12.13	67.0	791.0	400.5±34.75	26.0	791.0	204.6±16.02
SL	10.0	32.8	17.9±0.44	14.8	32.8	25.8±0.87	10.0	32.8	19.8±0.50
FL	11.0	33.9	19.3±0.45	15.5	36.2	27.9±1.00	11.0	36.2	21.4±0.54
TL	12.3	39.1	22.5±0.55	18.5	40.3	32.7±1.11	12.3	40.3	24.9±0.64
BD	3.1	10.5	5.8±0.15	4.5	11.2	8.4±0.31	3.1	11.2	6.4±0.17
SNL	4.7	17.9	10.0±0.28	8.4	20.5	15.1±0.53	4.7	20.5	11.2±0.31
EYD	6.6	12.8	9.8±0.12	9.2	15.0	11.3±0.22	6.6	15.0	10.2±0.12
HLT	7.4	63.2	33.4±0.97	32.9	69.0	51.4±1.57	7.4	69.0	37.6±1.10

Table 2 *(continue)*

	Male			Female			Both sexes		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
LBD	24.2	69.4	40.6±0.97	32.9	76.1	59.1±2.08	24.2	76.1	44.9±1.15
LPF	6.3	49.3	28.2±0.98	12.8	54.8	42.0±1.78	6.3	54.8	31.5±1.01
LPVF	19.4	53.7	31.1±0.79	25.6	53.3	44.8±1.48	19.4	53.7	34.3±0.88
LAB	6.3	42.6	19.7±0.70	15.7	44.3	28.7±1.16	6.3	44.3	21.9±0.70
BAD	4.4	21.8	8.8±0.37	5.8	25.2	12.3±0.67	4.4	25.2	9.6±0.35
DCP*	5.7	35.5	17.9±0.65	7.6	39.3	16.9±1.25	5.7	39.3	17.7±0.58
DRA	19.0	24.0		21.0	24.0		19.0	24.0	
PRA	12.0	20.0		10.0	20.0		10.0	20.0	
PVR*	7.0	10.0		9.0	17.0		7.0	17.0	
ARA	5.0	13.0		11.0	13.0		5.0	13.0	
K*	0.87	1.75	1.09±0.01	0.89	1.24	1.06±0.02	0.87	1.75	1.09±0.01

* No significant difference in the values between the sexes at 0.05 level
Legend:
Weight (WT), standard length (SL), forked length (FL), total length (TL), body depth (BD), snout length (SNL), eye diameter (EYD), head length (HLT), length of dorsal fin base (LBD), length of pectoral fin (LPF), length of pelvic fin (LPVF), length of anal fin base (LAB), length of base of adipose fin (BAD), depth of caudal peduncle (DCP), dorsal rays (DRA), pectoral rays (PRA), pelvic rays (PVR), anal rays (ARA) and condition factor (K)

in Anambra River, Nigeria (Nwani & Ude, 2005). Food availability and good water quality are synergist to healthy fish growth and maximum size a fish can attain. Time of sampling, sex and reproductive stage of the sampled fish might also be contributing factors to the differences observed.

The regression coefficients (b-values) obtained in this study depicted that male (2.90), female (2.97) and both sexes (0.92) of *D. engycephalus* had isometric growth since the values were not statistically different from 3 (Figures 5, 6 and 7). High values of co-efficient of determination (R^2) were obtained for male (0.97), female (0.98) and both sexes (0.98). The isometric growth pattern and condition factor reported in this study supported the results of Nwani (2006) on *D. engycephalus* from Anambra River. In similar study, Shinkafi et al. (2013) reported b-values of 2.29 (male), 2.48 (female) and 2.46 (both sexes) for *D. rostratus* in River Rima while in lower Nun River it was 2.76 for both sexes (Abowei, 2010). Result of condition factor ranged from 0.87-1.75 with a mean of 1.09±0.01 for both sexes. The condition factor (K) was not significantly higher (p>0.05) in male than female fish. This result was similar to that reported by Shinkafi et al. (2013) in River Rima. However, lower condition factor (0.98) was documented by Abowei (2010) for *D. rostratus* in lower Nun River. In River Anambra, condition factor for *D. rostratus, D. brevipinnis* and *D. engycephalus* were 1.12, 1.06, and 0.94 respectively (Nwani, 2006). This implied that aquatic ecosystem of Ogun River provided favourable aquatic environment that promoted good well-being of *D*. engycephalus. The observed sex ratio of male: female was 1:0.31 in favour of male production in the river system. Berte et al. (2008) reported sex ratio of 1:1.60 for *D. rostratus* in Bandama River, Ivory Coast.

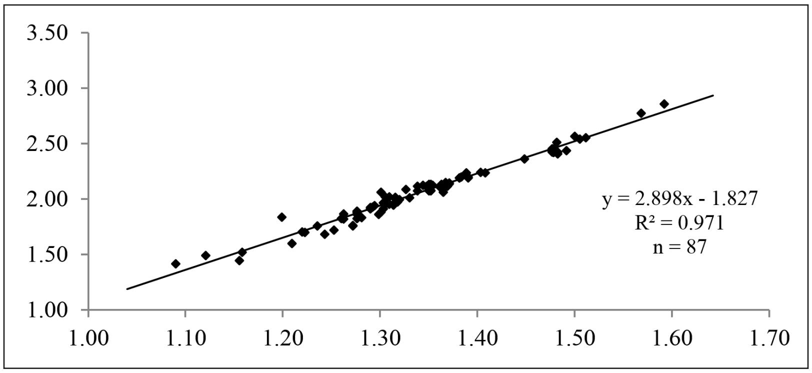

Figure 5. Length-weight relationship of male *Distichodus engycephalus* from middle basin of Ogun River, Southwest Nigeria

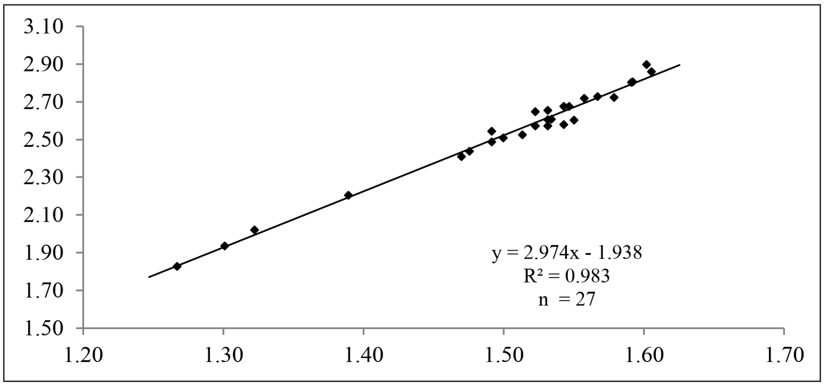

Figure 6. Length-weight relationship of female *Distichodus engycephalus* from middle basin of Ogun River, Southwest Nigeria

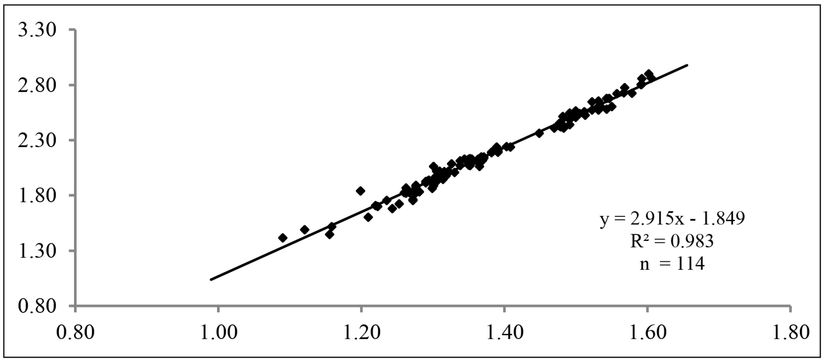

Figure 7. Length-weight relationship of *Distichodus engycephalus* (both sexes) from middle basin of Ogun River, Southwest Nigeria

Results of ANCOVA showed that there was significant difference in regression slopes of the sexes {*F(1, 111) = 0.01*} while adjusting for total length at 0.05 level, hence there is regression homogeneity between the sexes. Significant difference was observed in the investigated characters between male and female fish except in DCP and PVR. Student-test revealed that WT, SL, TL, BD, SNL, EYD, HLT, LDB, LPF, LPVF, LAB, BAD, DRA, PRA and ARA were significantly higher (p<0.05) in female than male fish. Countable characters are important in describing fish in that they may vary among and within the same species because they can be influenced by environmental factors especially at early stage of development. High positive correlations were observed in SL/TL (0.99), SL/FL (0.97) and FL/TL (0.98) as shown in Table 3. These results were expected as SL is part of the measurement of FL and TL. However, high correlations existed in TL/LPVF (0.98), SL/LPVF (0.97), SL/LBD (0.97), TL/LBD (0.97) and TL/WT (0.96). Zebe et al. (2010) documented TL/WT of 0.98 for *D. antonii* in Congo River. Least correlation value was obtained in DCP/HLT and DCP/LPF with 0.04 each. Generally, DCP had low relationship with the morphometric characters. The highest value (0.40) was observed in DCP/EYD.

Table 3
Correlation matrix of Distichodus engycephalus *from middle basin of Ogun River, Southwest Nigeria*

	WT	SL	FL	TL	BD	SNL	EYD	HLT	LDB	LPF	LPVF	LAB	BAD	DCP
WT	1													
SL	0.96	1												
FL	0.95	0.97	1											
TL	0.96	0.99	0.98	1										
BD	0.94	0.95	0.95	0.96	1									
SNL	0.90	0.93	0.92	0.94	0.90	1								
EYD	0.76	0.81	0.81	0.82	0.81	0.77	1							
HLT	0.90	0.92	0.93	0.92	0.88	0.91	0.75	1						
LBD	0.94	0.97	0.96	0.97	0.94	0.93	0.82	0.91	1					
LPF	0.77	0.74	0.82	0.79	0.77	0.77	0.63	0.80	0.78	1				
LPVF	0.94	0.97	0.95	0.98	0.94	0.93	0.79	0.92	0.96	0.80	1			
LAB	0.82	0.89	0.80	0.85	0.82	0.80	0.71	0.77	0.82	0.44	0.84	1		
BAD	0.62	0.71	0.59	0.66	0.60	0.59	0.53	0.56	0.63	0.13	0.63	0.86	1	
DCP	0.28	0.32	0.28	0.31	0.35	0.14	0.40	0.04	0.31	0.04	0.26	0.36	0.38	1

Weight (WT), standard length (SL), forked length (FL), total length (TL), body depth (BD), snout length (SNL), eye diameter (EYD), head length (HLT), length of dorsal fin base (LBD), length of pectoral fin (LPF), length of pelvic fin (LPVF), length of anal fin base (LAB), length of base of adipose fin (BAD) and depth of caudal peduncle (DCP)

CONCLUSION

It can be concluded that *D. engycephalus* in the middle basin of Ogun River is herbivorous, feeding mainly on Oscillatoria, Protococcus and Phormidium. The fish (male, female and both sexes) depicted isomerism in their growth pattern in the river system. The lotic environment of the river supported better growth performance of female fish. There were morphological differences between male and female fish which could be used to sex them, since they do not possess external reproductive features to differentiate the sexes. The study provides essential information needed for further studies on population dynamics and management of this fish species in aquatic ecological environments to ensure sustainable fish production.

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REFERENCES

Abobi, S. M. (2015). Weight-length models and relative condition factor of nine (9) fresh water fish species from the Yapei stretch of the White Volta, Ghana. *Journal of Zoology*, *79*(1), 30427-30431.

Abowei, J. F. N. (2010). Some population parameters of *Distichodus rostratus* (Günther, 1864) from the fresh water reaches of lower Nun River, Niger Delta, Nigeria. *Advance Journal of Food Science and Technology*, *2*(2), 84-90.

Adeyemi, S. O., Akombo, P. M., & Toluhi, O. O. (2009). Food and feeding habits of *Oreochromis niloticus* in Lake Gbedikere, Bassa, Kogi State. *Continental Journal of Animal and Veterinary Research*, *1*(1), 25-30.

Ahlbeck, I., Hansson, S., & Hjerne, O. (2012). Evaluating fish diet analysis methods by individual-based modelling. *Canadian Journal of Fisheries and Aquatic Sciences*, *69*(7), 1184–1201.

Arawomo, G. A. O. (1982). Food and feeding of three *Distichodus species* (Pisces: Characiformes) in Lake Kainji, Nigeria. *Hydrobiologia*, *94*(2), 177-181.

Ayoade, A. A., Sowunmi, A. A., & Nwachukwu, H. I. (2004). Gill asymmetry in *Labeo ogunensis* from Ogun River, Southwest Nigeria. *International Journal of Tropical Biology*, *52*(1), 171-175. doi: 10.15517/rbt.v52i1.14821

Bagenal, T. B., & Tesch, F. W. (1978). Age and growth. In T. Bagenal (Ed.), *Methods for the assessment of fish production in fresh waters* (pp. 101-136). London, England: Blackwell Scientific Publication.

Berte, S., Kouamélan, E. P., Ouattara, N. I., Koné, K., Goore, B. G., N' Douba, V., & Kouassi, N. J. (2008). Reproductive cycle and fecundity of *Distichodus rostratus* (Characiformes, Distichontidae) in a West African basin (Bandama River, Ivory Coast). *Tropicultura*, *26*(2), 104-107. doi: 10.19044/esj.2016.v12n24p157

Bolger, T., & Connolly, P. L. (1989). Selection of suitable indices for the measurement and analysis of fish condition. *Journal of Fish Biology*, *34*(2), 171-182. doi: 10111/j.1095-8649.1989.tb03300.x

Chipps, S. R., & Garvey, J. E. (2007). Assessment of food habits and feeding patterns. In *Analysis and interpretation of freshwater fisheries data* (pp. 473-514). Bethesda, USA: American Fisheries Society.

Froese, R. (2006). Cube law, condition factor and weight-length relationships: History, meta-analysis and recommendations. *Journal of Applied Ichthyology*, *22*(4), 241- 253.

King, M. (2007). *Fisheries biology, assessment and management*. Cambridge, United Kingdom: Blackwell Science Limited.

Kulbick, M., Guillemot, N., & Amand, M. (2005). A general approach to length-weight relationships for New Caledonian lagoon fishes. *Cybium*, *29*(3), 235-252.

Mendes, B., Fonseca, P., & Campos, A. (2004). Weight-length relationship for 46 fish species of the Portuguese west coast. *Journal of Applied Ichthyology*, *20*(5), 355-361. doi: 10.1111/j.1439-0426.2004.00559.x

Moutopoulos, D. K., & Stergiou, K. I. (2002). Length-weight and length-length relationship of fish species from the Aegean Sea (Greece). *Journal of Applied Ichthyology*, *18*(3), 200-203.

Nwani, C. D. (2006). Length-weight relationship and condition factor of *Distichodus* species of Anambra River. *Animal Research International*, *3*(2), 461-465.

Nwani, C. D., & Ude, E. F. (2005). Morphometric variations among three *Distichodus* species of Anambra River, Nigeria. *Animal Research International*, *2*(3), 372-376. doi:10.4314/ari.v2i3.40872

Oketola, A. A., Osibanjo, O., Ejelonu, B. C., Oladimeji, Y. B., & Damazio, O. A. (2006). Water quality assessment of River Ogun around the cattle market off Isheri, Nigeria. *Journal of Applied Sciences*, *6*(3), 511-517.

Shinkafi, B. A., Salim, A. M., & Yusuf, M. A. (2013). Some aspects of the biology of *Distichodus rostratus* (Günther, 1864) in River Rima, North-western Nigeria. *Greener Journal of Biological Sciences*, *3*(4), 136-145.

Sinovcic, G., Franicvic, M., Zorica, B., & Ciles-Kec, V. (2004). Length-weight and length-length relationships for ten pelagic fish species from the Adriatic Sea (Croatia). *Journal of Applied Ichthyology*, *20*(2), 156-158. doi: 10.1046/j.1439-0426.2003.00519

Sokal, R., & Rohlf, F. J. (1998). *Biometry*. New York, NY: William Freeman and Company.

Sparre, P., & Venema, S. C. (1992). *Introduction to tropical fish stock assessment. Part 1. Manual. FAO Fisheries Technical paper 306/1 revision 1*. Rome, Italy: Food and Agriculture Organization.

Teugels, G. G., Mocgreid, G., & King, R. P. (1992). *Fishes of the Cross River basin (Cameroon-Nigeria): Taxonomy, zoogeography, ecology and conservation*. Tervuren, Belgium: Royal Museum for Central Africa.

Thomas, J., Venu, S., & Kurup, B .M. (2003). Length-weight relationship of some deep sea fish inhabiting the continental slope beyond 250m depth along the west coast in India. *NAGA, World Fish Center Quarterly*, *26*(2), 17-21.

Zar, J. H. (1984). *Biostatistical analysis*. Upper Saddle River, USA: Prentice Hall.

Zebe, V. M., Micha, J. C., Moreau, J., & Bekeli, M. N. (2010). Age and growth of *Distichodus antonii* (Schilthuis, 1891) (Pisces, Teleostei, Distichontidae) in pool Malebo, Congo River. *African Journal of Environmental Science and Technology*, *4*(5), 279 -283.

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Lethal Doses and Histopathological Changes in Liver and Kidney of Healthy *Clarias gariepinus* Sub-adult Exposed to Red *Allium cepa* Linn. Bulb

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ABSTRACT

Adverse effects of *Allium cepa* bulb has been well overlooked because it is natural and assumed safe. This study investigated the lethal doses and histopathological changes in liver and kidney of healthy *Clarias gariepinus* sub-adult exposed to red *Allium cepa* bulb at varying concentrations (200, 150, 100, 50 and 25 g/kg) of *A. cepa* via diet and bath (5, 3, 1.5, 0.7 and 0.4 g/L) for two weeks. Specimens were sacrificed, liver and kidneys collected, processed and examined for histopathological changes. Proximate analysis, qualitative and quantitative phytochemistry was determined using standard methods. Brine Shrimp lethality assay (BSLA), LD_{50} and LC_{50} of the onion on experimental fish were investigated. Saponins, tannins, phenols, flavonoids and alkaloids were present. LC_{50} of the onion extract was between 0. 51 mg and 0.64 mg in the BSLA while mean LD_{50} and LC_{50} for dietary and bath treatments were 447.1 g/kg and 12.2 g/L. Liver histopathology showed vacuolar degeneration of the hepatocytes and congestion of central vein, while necrosis of the epithelial cells and

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haemosiderosis were observed in the kidney at high concentrations. *Allium cepa* is safe in fish when administered in lower dose either through diet or bath exposure but was toxic at high dose.

Keywords: *Allium cepa*, *Clarias gariepinus*, histopathology, phytochemistry, toxicity

INTRODUCTION

The use of medicinal plants for human and animal therapy and production performance had been on the rise for decades in which herbal medicine is said to be popular among 70% of the population (Batta, 2012). This is due amongst other reasons to the resistance problems associated with the use of conventional drugs (Nebedum et al., 2009). *Allium cepa* Linn. (onion) is a commonly grown and is second most consumed vegetable in the world (Kuete, 2017). Aside its nutritive values, it has been used for folklore medicine for the treatment of different infections and diseases ranging from trauma, convulsion, hay fever, pneumonia and much more (Patil & Patil, 2007).

Allium cepa had been well documented to have a wide range of therapeutic potentials (Augusti, 1996; Griffiths et al*.,* 2002). It has been reported that variable concentrations of the *A. cepa* bulb administered to fish either via diet and bath improved the growth performance, feed utilization, body composition and survival of different fish species (Obaroh et al., 2018; Saleh et al., 2015). It has also been said to have antimicrobial (Amrevuawho et al., 2016; Bello et al., 2012) and immunostimulatory activities (Saleh et al. 2015). Other studies using other animal models and *in vitro* have also confirmed the antioxidant (Ashwini et al., 2013), antihepatotoxic (Obioha et al., 2009), antilipidemic (Ugwu & Olam, 2011) and anticancer (Nicastro et al., 2015) effects of the onion bulb. However, despite reports that administration of high dose resulted to mortality in experimental animals (Borelli et al., 2009; El-Sayed et al., 2015; Salami et al., 2012), there is dearth of information on the safe dose and pathological effects of *A. cepa* on the internal organs of fish.

Fisheries and aquaculture are important means of livelihood to many farmers and have contributed to the economy of many countries (Martini & Lindberg, 2013). In Nigeria, agriculture contributed 26.15% to GDP in the fourth quarter of 2018 (National Bureau of Statistics [NBS], 2019) to which the fisheries subsector contributes 3-4% (Food and Agricultural Organization [FAO], 2019). *Clarias gariepinus* Burchell is the most cultured fish species in the study area because of its hardiness, adaptability and acceptability. However, in a bit to increase fish production, intensification of the aquaculture sector has led to increased issues of disease outbreak and huge economic loss in this subsector (Opiyo et al., 2018). Hence this study determined the concentration of the *A. ceepa* bulb that can be recommended for use as feed additive and bath treatment to enhance the performance of *C. gariepinus*.

MATERIALS AND METHODS

Experimental Fish

Five hundred sub-adult *C. gariepinus* of average weight 421±2.41g were obtained from a reputable fish farm within Ogun State, Nigeria. Handling during transport was well managed to reduce stress and transportation of fish was in 120 L bowls to experimental site. Fish were distributed into 50 pieces of 80 L bowls at 10 fish/bowl for 14 days for acclimatization. Fish were fed 3 mm commercial diets "Coppens" (Alltech Coppens, Netherlands) twice daily within this period (Table 1). Water quality parameters of temperature (28-32^{o}C), dissolved oxygen (4-6.5 mg/L) and pH (6.5-9) were managed at optimal range by daily change of water and monitored using HANNA multi parameter (Model HI98194) water test kit. Health status of experimental fish was evaluated before commencement of the study by physical observation of the fish activities such as swimming pattern, response to feed, fish skin condition according to the method of Johansen et al. (2006). No clinical sign of disease was observed.

Table 1
Manufacturers' proximate composition of 3 mm Coppens feed

Major components	% composition
Crude protein	45
Crude fat	12
Crude fiber	1.5
Ash	9.5
Moisture content	8.3

Collection, Preparation and Extraction of Plant Bioactive Compounds

Fresh onion bulbs (red variety) were purchased from the local onion market in Abeokuta, peeled, washed using clean water, drained properly and blended using manual blender to obtain a coarse blend. The blended onion (1000 grams) was macerated in different polar solvents: n-hexane, acetone and methanol at a ratio of 1 kg: 1.3 L of solvent and placed in an electronic flask shaker (SM/DR-10, Singifield Medicals, England) for 15 hours. Extraction was by cold maceration method, sieved using Whatman paper (125 cm pore size) and concentration was by the use of a rotary evaporator (Mode: HEI-VAP SILVER, Heidolph Instruments GmbH & Co. KG, Germany) at 40^{o}C to obtain the crude extract. Crude extracts from each solvent were weighed and refrigerated at 4^{o}C for a two day period before commencing of study. Identification and authentication of plant were done at the Department of Forestry and Wildlife Management Herbarium, Federal University of Agriculture, Abeokuta (FUNAAB) and voucher specimen/ID number UAHA: 018/0001 given.

Phytochemical Screening

The phytochemical composition of the onion bulb was determined using standard methods outlined by Harborne and described by Santosh et al. (2013).

Fish Diet Preparation

Pearson square method of fish feed formulation was adopted in the formulation of six experimental diets containing 40% crude protein as described by Adegbesan et al. (2017). The fresh *A. cepa* bulb meal was incorporated into each of the diet at 0 (control), 2.5, 5, 10, 15 and 20%.

Proximate Analysis of *Allium cepa* and Experimental Diets

The proximate compositions of whole *A. cepa* bulb and experimental diets were carried out as follows: for crude fibre, moisture and fat contents, the method described by the Association of Analytical Chemist (AOAC) (2005) was employed using n-hexane as solvent. The Macro Kjeldahl method of Kirk et al. (1991) was adopted for crude protein and total ash contents determination while percentage carbohydrate content was by differentiation.

Experimental Design for Sub Chronic Toxicity Studies

For sub chronic toxicity studies, complete randomized design was adopted. Experimental fish were grouped into A, B and C. Group A served as dietary treatment group with onion inclusion levels 20, 15, 10, 5 and 2.5% in experimental diets. Group B fish on the other hand were exposed to bath treatment with varying concentrations of 5, 3, 1.5, 0.7 and 0.4 g/L of the onion bulb. Group C was not exposed to any form of onion and served as control. Fish were replicated thrice per treatment with 15 fish/replicate. Static renewal bioassay system was adopted in this study to maintain a constant onion concentration. Pilot study for the bath treatment was conducted prior to commencing of study. In the pilot study, the onion bulb slurry was introduced into the fish experiment at 20, 15, 10 and 5 g/L. This was done to ascertain the range of concentrations that will not kill the experimental fish immediately. Mortality was monitored and recorded.

Experimental protocols adopted in this study for test fish were according to the internationally accepted standard for the laboratory animal usage. They were approved by Ethics Committee on the Laboratory Animal Use of the College of Veterinary Medicine, FUNAAB.

In vitro Assessment of Toxicity of the Onion Bulb

Brine Shrimp Lethality Assay (BLSA). Using the assay system, 5 Petri dishes were prepared. Each of which contained 20 ml of seawater (35 ppt) well filtered. Two-fold dilution was set up to get different concentrations (2, 1, 0.5, 0.25, and 0.125 mg/ml) of the plant extracts.

For hatchability assay, hatching success of *Artemia salina* cysts in the various petri dishes containing different concentrations of plant extracts and positive control was evaluated using the method of Manilal et al. (2009). Ten (10) individuals of *A. salina* cysts were introduced into each petri dish containing 20 ml of filtered seawater. Petri dishes were covered partly and placed in the incubator at 28°C for 24- 72 hours under steady illumination. After every 24 hours, free nauplii in each petri dish were counted. Hatchability percentage was calculated by comparing the hatched nauplii in individual petri dish to the total number of cysts stocked (Carballo et al., 2003). Minimum Inhibitory Concentration (MIC) was then determined.

Method of Data Analysis. Complete randomized design was adopted for this assay with 2 replicate incubations per treatment. Mortality data (comprising of *A. salina* that hatched out and died and unhatched cyst) obtained from the different concentrations extracts and control experiments were used to plot dose/response. Their respective LC_{50} values were then determined (Syahmi et al., 2010). LC_{50} values were determined using the probit dose-response curve (Finney, 1952).

In vivo Assessment of Toxicity of the Onion Bulb in *Clarias gariepinus*

Determination of Lethal Dose of *Allium cepa* Slurry on Experimental Fish. The LD_{50} was determined by log-dose/probit regression live method (Finney, 1952). Experimental fish were exposed to 200, 150, 100, 50 and 25 g/kg diets and 5, 3, 1.5, 0.7 and 0.4 g/L of onion bulb via prolonged bath. Experimental fish were observed for 14 days and then mortality was noted for further calculation. Graph empirical probit and log dose was then plotted and LD_{50} calculated using regression analysis

Histopathology

Three fish each were selected from the treated groups and the control. The livers and kidneys from each of these fish were preserved in buffered formalin at 10% concentration. The tissues were trimmed and different concentrations of alcohol (50, 70 80, 90, 100%) was used for dehydration, tissues were then cleared using xylene and paraffin wax used for embedding. Section of 5µm were cut and stained with haematoxylin and eosin according to the method described by Pulvertaft (1950). Sections were then examined under the light microscope.

Data Analysis

All obtained data from the study were expressed in mean ± standard deviation (mean ± SD). One-way analysis of variance (ANOVA) was used to test for the means. Means were then separated using Duncan Multiple Range Test (Duncan, 1955). Probability levels < 0.05 were significant. The LD_{50} and LC_{50} were analyzed for using Probit linear regression Finney (1952).

RESULTS AND DISCUSSION

Proximate Analysis of the Onion Bulb

Proximate analysis of the onion bulb showed high moisture content (83.28%) followed by percentage crude protein (8.48%) and carbohydrate (5.94%) (Table 2). Most of the phytochemicals present in the bulb such as proteins and carbohydrates have good nutritional values. For instance Bhattacharjee et al. (2013) in their study posited that the *A. cepa* plant stored up their carbohydrate as fructans which explained the prebiotics activity of the bulb in the gut of fish thereby conferring on it, its growth promoting activity. Available evidences also suggest that fructooligosaccharide and inulin

Table 2
Proximate analysis of whole Allium cepa *bulb*

Moisture content (%)	Fat content (%)	Ash content (%)	Crude Fiber (%)	Crude Protein (%)	Carbohydrate (%)
83.28 ± 0.01	0.14 ± 0.00	0.5 ± 0.01	1.68 ± 0.01	8.48 ± 0.01	5.94 ± 0.05

present in the onion bulb, acts as prebiotic and influence different physiological activities of fish, such as blood and serum parameters, general performance of the fish including nutrient utilization and body composition, lipids breakdown and blood cholesterol levels (Yarahmadi et al., 2016).

Proximate Analysis and Percentage Composition of the Experimental Diets

Table 3 revealed the mean values and percentage gross composition of the diets used for both experimental and control fish. Crude protein content in all the experimental diets 1,2,3,4,5 and control diets were 40.3±0.06, 40.9±0.02, 40.8±0.11, 40.6±0.10, 40.2±0.02 and 40.0±0.27 respectively. Highest carbohydrate percentage was obtained in diet 1 (40.68±0.67) and least in diet 2 (34.48±0.11).

Phytochemical Analysis of *Allium cepa*

Phytochemical analysis of *A. cepa* extract showed various chemical compounds in

Table 3
Proximate analysis and Percentage composition (%) of the experimental diets

Parameters	1	2	3	4	5	6
Moisture content (%)	4.66±0.06	3.28±0.03	3.94±0.04	3.58±0.088	3.71±0.021	4.25±0.023
Fat (%)	2.50±0.03	6.13±.05	5.19±.06	6.01±.05	5.33±0.01	4.51±0.04
Ash (%)	9.80±0.03	12.20±0.02	11.38±0.02	12.14±0.06	11.88±0.02	10.89±0.02
Crude fiber (%)	2.06±0.07	3.01±0.02	2.46±0.07	2.81±0.06	2.66±0.03	2.18±0.1
Crude Protein (%)	40.3±0.06	40.9±0.02	40.8±0.11	40.6±0.10	40.2±0.02	40.0±0.27
Carbohydrate (%)	40.68±0.67	34.48±0.11	36.23±0.31	34.86±0.19	36.22±0.22	38.17±0.07
Fish meal	23	23	23	23	23	23
Soybean meal	45	45	45	45	45	45
Maize	30	30	30	30	30	30
Fish premix	0.5	0.5	0.5	0.5	0.5	0.5
Dicalciumphosphate	1	1	1	1	1	1
Toxin binder	0.1	0.1	0.1	0.1	0.1	0.1
Salt	0.2	0.2	0.2	0.2	0.2	0.2
Lysine	0.1	0.1	0.1	0.1	0.1	0.1
Methionine	0.1	0.1	0.1	0.1	0.1	0.1
Onion	20	15	10	5	2.5	0

Key: 1 = experimental diet with *A. cepa* inclusion of 200g/kg, 2 = experimental diet with *A. cepa* inclusion of 150g/kg, 3 = experimental diet with *A. cepa* inclusion of 100g/kg, 4 = experimental diet with *A. cepa* inclusion of 50g/kg, 5 = experimental diet with *A. cepa* inclusion of 25g/kg, 6 = control diet with 0g/kg of *A. cepa* inclusion different composition in which combined anthraquinones had the highest mean value (1.52 ±0.04) while steroids had the least (0.016 ± 0.00) (Table 4). Saponins, flavonoids, alkaloids, phenol, steroids, tannins and cardiac glycosides present in the onion bulb in this study corroborated the study by Ogbonna et al. (2016). Although, Gazuwa et al. (2013) reported the presence of alkaloid, flavonoid, cardiac glycoside and steroid, they however observed that tannins, saponins and total phenols were absent in the fresh onion bulb which was not in agreement with the present study. The difference may be due to cultivar, environment/geographical location, maturity stage, storage time and agronomic conditions (Abayomi & Terry, 2009).

Table 4
Phytochemistry of the onion bulb

S/N	PARAMETERS	QUANTITATIVE %	QUALITATIVE
1	Alkaloid	0.74 ± 0.01	+
2	Flavonoid	0.45 ± 0.01	+
3	Tannin	0.96 ± 0.01	++
4	Saponin	0.27 ± 0.01	+
5	Glycosides	0.55 ± 0.00	+
6	Total phenol	0.97 ± 0.01	++
7	Steroid	0.016 ± 0.00	+
8	Free anthraquinone	0.30 ± 0.00	+
9	Combined anthraquinone	1.52 ±0.04	++

Note. + = presence minimal, ++ = highly present

Brine Shrimp Assay

Hatching success of *A. salina* in the different solvent extracts is represented in Table 5. Hatchability percentage was observed to significantly increase ($P > 0.05$) with length of incubation. With highest hatchability 75% recorded for methanolic extract and least 65% in n-hexane extract. The minimum inhibitory concentration (MIC) was lowest in acetone extract. The mean lethal concentration of all the extracts showed concentrations less than 1mg/ml. Finney probit dose-response graph revealed 'b' values of 2.428, 2.288 and 2.348, 'a' values of 6.229, 6.453 and 6,451 and 'r' calculated was 0.95, 0.88 and 0.97 respectively for methanol, acetone and n-hexane extracts of the onion bulb (Table 5). *Allium salina* cysts hatchability percentage was higher in the methanol extract. However, this was not the result with the LC_{50} value obtained for the methanol extract which indicated higher bioactivity. This finding disagreed with the reports by Ohikhena et al. (2016). They documented that hatchability percentage in the acetone extract of *Phragmanthera capitata* was higher than in the methanol extract which could be attributed to the differences in the test plant species.

Solvent-dependent MIC that was obtained in the BSLA could be attributed to the polarity of the solvents of extraction

Table 5
Dose-response, Percentage hatchability, MIC and LC_{50} of Brine shrimp exposed to Allium cepa *using probit*

Extracts	Dose mg/ml	Total nauplii	Live nauplii	Hatchea-bility (%)	MIC (mg/ml)	Log dose	Mortality	Mortality %	Probit	LC_{50} (mg/ml)	R value
FOBM	2	20	0	0	2	0.3010	20	100	7.44	0.5113	0.9476,
	1	20	4	20		0	16	80	5.84		
	0.5	20	9	45		-0.3010	11	55	5.13		
	0.25	20	12	60		-0.6021	8	40	4.75		
	0.125	20	15	75		-0.9031	5	25	4.33		
FOBA	2	20	1	5		0.3010	19	95	6.64	0.6350	0.8775
	1	20	0	0	1	0	20	100	7.44		
	0.5	20	7	35		-0.3010	13	65	5.39		
	0.25	20	11	55		-0.6021	9	45	4.87		
	0.125	20	14	70		-0.9031	6	30	4.48		
FOBH	2	20	0	0	2	0.3010	20	100	7.44	0.6180	0.9742
	1	20	2	10		0	18	90	6.28		
	0.5	20	6	30		-0.3010	14	70	5.52		
	0.25	20	11	55		-0.6021	9	45	4.87		
	0.125	20	13	65		-0.9031	7	35	4.61		

Key: FOBM – Fresh onion bulb methanol extract, FOBA – Fresh onion bulb acetone extract, FOBH – Fresh onion bulb n-hexane extract

resulting in varying extraction capability. Various studies have shown that solvent polarity determines the phyto compound extracted from a plant (Illoki-Assanga et al., 2015; Widyawati et al., 2014). The result obtained for BSLA of various extracts of the onion bulb suggests that it has minimal toxicity according to the interpretation given by Bastos et al. (2009). Hence, these extracts may be considered safe for use as feed additive but care should however be taken as toxicity could be conferred with high concentration or long duration of exposure. The mild toxicity effect of the onion bulb extract is an indication that the bulb of onion could be used as an alternative in treatment and management of disease conditions, since BSLA is used to indicate presence and level of bioactivity of a plant (Ohikhena et al., 2016).

Clinical Signs of *Allium cepa* at High Concentrations

No clinical signs were observed in sample fish after 14 days of exposure to the onion bulb slurry through fish diets ad bath.

Gross Lesions observed in *Clarias gariepinus* at Postmortem

Paleness of gills, eyes were opaque at 5 mg/ml concentrations of bath treatment. Skin lesions were also observed in sample fish especially at 200g/kg diet treatments. Mortality was highest in 200g/kg diet (Table 6).

Lethal Concentration of *Allium cepa* on Experimental Fish

The minimum lethal dose and concentration of *A. cepa* in *C. gariepinus* were 441.7g/kg for those given as dietary inclusion and 12.2g/L for those used as bath (Table 6). The LC_{50} for experimental fish was low in fish exposed via bath. Finney probit dose-response graph revealed 'b' values of 4.86 and 4.34, 'a' values of -7.71 and +1.62, 'r' calculated was 0.81 and 0.91 respectively for feed and bath exposure to the onion prophylaxis. The 'r' value showed a strong positive relationship between concentration and mortality in both routes of administration.

The lethal dose and concentration calculated for the onion bulb on *C. gariepinus* implied that lower dosage of the plant is considered safe for fish. The mortality and other clinical signs recorded in fish exposed to higher concentration of the onion bulb slurry in both bath and diet treatments shows that only lower dose could be used as feed additive. Although, few controlled and clinical studies have been conducted on the safety of fresh onion bulb in aquaculture, the findings of the present study suggest that safety could be dose-dependent, thus, at higher concentration; the onion bulb may not be safe. Strong positive relationship observed from the regression analysis in both BSLA and LD_{50} between concentration and percentage mortality further confirms the dose-effect potential and toxicity likely with increase concentration of the onion bulb.

Histopathology

The kidneys and livers of fish treated with *A. cepa* revealed varying degrees of vacuolar degenerations. The liver had vacuolar degeneration and necrosis of hepatocytes, congestion of the central veins and haemochromatosis in hepatocytes. The kidneys had vacuolar degeneration of tubular and glomerular epithelial cells (Table 7 and Figures 1 and 2). However, degenerations were more pronounced in bath treatments than feed.

Table 6
Lethal dose and lethal concentration of Allium cepa *slurry on experimental fish*

Treatments	Dose mg/ml	Log dose	Mortality	Mortality %	Probit	LC_{50}	R value
Dietary inclusion	200	2.3010	11	23.3	4.48	441.7 g/kg	0.8124
	150	2.1761	5	10	3.72		
	100	2	0	0	0		
	50	1.6989	0	0	0		
	25	1.3979	0	0	0		
Bath	5	0.6989	9	20	4.29	12.2g/L	0.9149
	3	0.4771	5	10	3.72		
	1.5	0.1761	5	10	3.72		
	0.7	-0.1549	0	0	0		
	0.4	-0.3979	0	0	0		

The effect of the onion bulb administered orally and through bath on the liver and kidney was determined in the study. The mild to moderate changes observed in these organs are reversible (Ozkurt et al., 2014). This suggests that the onion bulb has minimal detrimental effect when administered at low concentration. The degenerative changes observed in these organs might have resulted due to the presence of the phytochemical anthraquinone in the bulb (Chan & Lin, 2009).

Mild degenerations observed in *C. gariepinus* liver exposed to various concentrations of the fresh onion bulb via dietary inclusion and bath relative to the control liver confirmed the tendency for toxicity of the onion bulb (Borelli et al., 2009; Parton, 2000). Degenerative changes in the hepatocytes of the treatment group could be due to the presence of phytocompounds in the bulb of the onion which have been reported to have pathological responses in man (Abalaka et al., 2015). It could

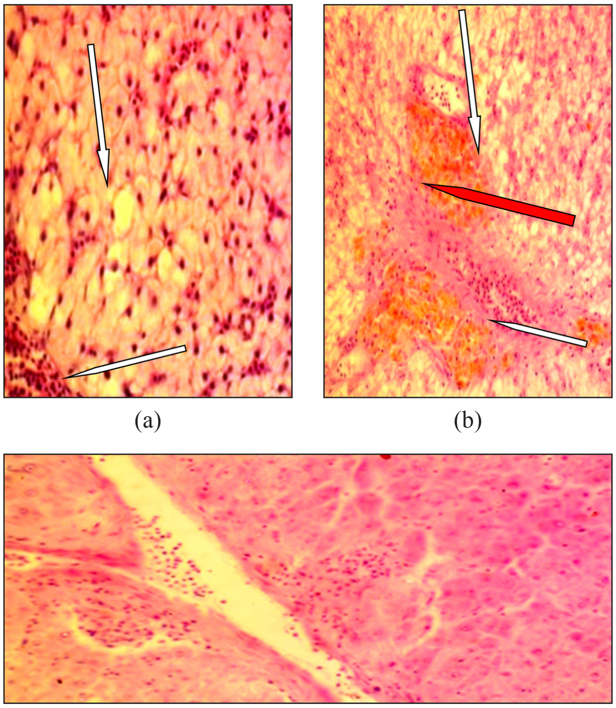
Table 7
Histopathological changes observed in the organs of Clarias gariepinus sub-adult exposed to varying concentrations of whole Allium cepa *through feed and water*

	Histological signs	Route of administration										
		Feed (inclusion levels of onion in g/kg)					Bath (onion inclusion levels in g/L)					Control
		200	150	100	50	25	5	3	1.5	0.7	0.4	0 g/L
Kidney	Degeneration and necrosis of tubular epithelial cells	-	+	+	+	+	++	++	++	++	+	-
	Degeneration and necrosis of glomerular epithelial cells	-	+	+	+	+	+	+	+	+	+	-
	pigmentation of tubular epithelial cells	-	-	-		-	-	+	+	+	+	-
	haemosiderosis in the kidney	-	-	-		-	+	+	+	+	+	-
Liver	Vacuolar degeneration of the hepatocytes	+	++	++	++	++	++	+	++	++	+	-
	Congestion of the blood vessels	-	+	-	-	-	-	+	-	-	+	-
	Congestion of the central vein	-	+	-	+	-	+	+	+	+	+	-
	Necrosis of hepatocytes	-	-	-	-	-	-	-	-	-		-
	Hepatocytes infiltration by monoclear cells	-	-	-	-	-	-	-	-	-	-	-
	Haemosiderosis	-	-	-	-	-	-	-	+	+	-	-
	Encapsulation by fibrous connective tissue	-	-	-	-	-	-	-	-	-	-	-

Key: ++: = Present and distinct with morphological changes of histological signs on the organ and tissues. +=Present but less marked (mild) than usual. - = No lesion and morphological changes in organ and tissues also be due to inability of the liver cells to metabolize fat during digestion resulting to small vacuoles of fat accumulating in the cytoplasm. However, vacuolar degeneration is reversible and mild degenerations may have no effect on cell function (Marcon et al., 2015).

The findings of this study corroborated the works of Al-Salahy and Mahmoud (2003) who reported hepatic vacuolar degeneration and necrosis in *Chrysichthys auratus* exposed to various concentrations of *Allium sativum* orally. However, Amrevuawho et al. (2016), reported on the ability of the onion bulb extracts to restore damaged liver cells of *C. gariepinus* exposed to *Pseudomonas aeruginosa*. Reason for these differences could be attributed to the state of health of the experimental fish.

The kidneys of treated experimental fish in this study showed various degrees of degeneration and necrosis of the tubular and glomerular epithelial cell in both routes of administration. The presence of haemosiderin laden macrophages was more in the fish exposed through bath. This might be due to direct accessibility of the extract to the circulatory system of the fish (blood)



(a) (b)

(c)

Figure 1. Liver sections in both routes of administration and control
Note. a. Section of the liver of fish treated orally with *A. cepa* showing severe vacuolar degeneration of hepatocytes (arrow) and congestion of sinusoid and central vein (arrow head) b. T8 Section of the liver of fish treated through bath with *A. cepa* showing vacuolar degeneration (arrow) and pigmentation of the hepatocytes (red arrow head) and congestion of the central vein (arrow head) c Section of the liver of fish not treated with *A. cepa* appearing apparently normal (x400; H 7 E)

via the gills during prolong bathing and subsequently enhance direct access to the kidney. Unlike group A in which dietary method was used, it was possible that most of the chemical compounds in the extract might have undergone biodegradation by the intestinal enzymes before entering the liver and the kidney resulting to mild effect in this group. Ola-Mudathir and Maduagwu (2014) and Wani et al. (2018) reported a contrary finding to that in this present study. Reasons could be due to differences in laboratory animal used and also could be due to the state of the animals.

Damage to tubular epithelial cells and haemosiderosis observed in the kidney can rarely result in kidney dysfunction and could be reversed (Ozkurt et al., 2014; Relia & Kaushik, 2010; Yatmark et al., 2016). Haemosiderosis could also be inherited as reported by Ozkurt et al. (2014) thus it could not be conclusive from this study that these degenerations were caused by the intake of the onion bulb.

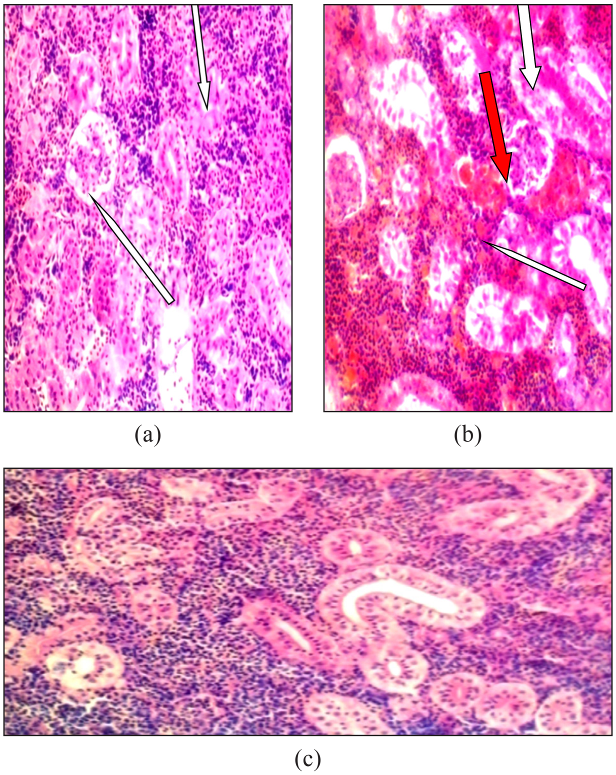


Figure 2. Comparative kidney sections in both routes of administration and control
Note. a. Treatment 3(100g/kg) Section of the kidney of fish treated with *A. cepa* showing degenerations and necrosis of tubular (arrow) and glomerular epithelial cells (arrow head) b. Treatment 6 (5g/L) Section of the kidney of fish treated with *A. cepa* show degeneration and necrosis of tubular (white arrow) and glomerular (red arrow) epithelial cells (arrow) with haemochromatosis (white arrow head) c. Section of the kidney of fish not treated with *A. cepa* appearing apparently normal (x400; H & E)

CONCLUSION

Allium cepa is safe in fish when administered in lower dose either through diet or bath exposure, since degenerative changes are reversible; however, the onion bulb extract can be harmful at high dose due to necrosis of hepatocytes and tubular epithelial cells.

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REFERENCES

Abalaka, S. E., Fatihu, M. Y., Ibrahim, N. D. G., & Ambali, S. F. (2015). Liver histopathological changes in *Clarias gariepinus* exposed to ethanol extract of *Adenium obesum* stem bark. *Journal of Morphological Sciences*, *32*(1), 22-28. doi: 10.4322/jms.069314

Abayomi, L. A., & Terry, L. A. (2009). Implications of spatial and temporal changes in concentration of pyruvate and glucose in onion (*Allium cepa* L.) bulbs during controlled atmosphere storage. *Journal of Science Food and Agriculture*, *89*(4), 683-687. doi: 10.1002/jsfa.3502

Adegbesan, S. I., Obasa, S. O., & Abdulraheem, I. (2017). Metabolic activities and health indices of African catfish (*Clarias gariepinus*) fed varying levels of *Zingiber officinale* root. *Journal of Applied Biology and Biotechnology*, *5*(4), 21-28.

Al-Salahy, M. B., & Mahmoud A. B. (2003). Metabolic and histological studies on the effect of garlic administration on the carnivorous fish *Chrysichthys auratus*. *Egyptian Journal of Biology*, *5*(1), 94-107.

Amrevuawho, M. O., Akinyemi, A. A., Oyewusi, A. J., Bankole, O. M., & Ezeri, G. N. O. (2016). Effects of onion (*Allium cepa*) and chloramphenicol on haematological parameters, histopathology and survival of catfish C*larias gariepinus* (Burchell, 1822) sub-adult infected with *Pseudomonas aeruginosa*. *Journal of Veterinary Science and Technology*, *11*(1), 1-12.

Association of Official Analytical Chemists. (2005). *Official methods of analysis of the Association of Analytical Chemists International* (18th ed.). Washington, USA: MD Publishing.

Augusti, K. T. (1996). Therapeutic values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.). *Indian Journal of Experimental Biology*, *34*(7), 634-40. doi: 10.19046/abp.v03i04.01

Ashwini, M., Balaganesh, J., Balamurugan, S., Murugan, S. B., & Sathishkumar, R. (2013). Antioxidant activity in *in vitro* and *in vivo* cultures of onion varieties (Bellary and CO 3). *Food and Nutrition Sciences*, *4*, 918-923. doi: 10.4236/fns.2013.49119

Bastos, M. L. A., Lima, M. R. F., Conserva, L. M., Andrade, V. S., Rocha, E. M. M., & Lemos, R. P. L. (2009). Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae), extracts and their main constituents. *Annals of Clinical Microbiology and Antimicrobials*, *8*(1), 16. doi: 10.1186/1476-0711-8-16

Batta, H. (2012). Press coverage of traditional medical practice in Nigeria. *Journal of Communications*, *3*(2), 75-89.

Bello, O. S., Olaifa, F. E., Emikpe, B. O., & Ogunbanwo, S. T. (2012). The effect of walnut (*Tetracarpidium conophorum*) leaf and onion (*Allium cepa*) bulb residues on the tissue bacteriological changes of *Clarias gariepinus* juvenile. *Bulletin des Santé et Production Animales en Afrique*, *60*(2), 205-212.

Bhattacharjee, S., Sultana, A., Sazzad, M. H., Islam, M. A., Ahtashom, M., & Asaduzzaman, M. (2013). Analysis of the proximate composition and energy values of two varieties of onion (*Allium cepa* L.) bulbs of different origin: A comparative study. *International Journal of Nutrition and Food Sciences*, *2*(5), 246-253. doi:10.11648/j.ijnfs.20130205.16

Borelli, V., Lucioli, J., Fernando, H. F., Giovana, H. P., Fleck, R. J., Davi, T. S., & Aldo, G. (2009). Fatal onion (*Allium cepa*) toxicosis in water buffalo (*Bubalus bubalis*). *Journal of Veterinary Diagnostic Investigation*, *21*(3), 402-405. doi: 10.1177/104063870902100321

Carballo, J. L., Hernández-Inda, Z. L., Pérez, P., & García-Grávalos, M. D. (2003). A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. *BMC Biotechnology*, *2*(1), 17-21. doi: 10.1186/1472-6750-2-17

Chan, K., & Lin, T. X. (2009). Chapter 48 - Treatments used in complementary and alternative medicine. *Side Effects of Drugs Annual*, *31*, 745-756. doi: 10.1016/S0378-6080(09)03148-1

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics*, *11*(1), 1-42.

El-Sayed, Y. S., El-Okle, O. S. M., Hassan, S. M. H., & Bakir, N. M. A. (2015). Poisoning of cattle feeding on Allium ampeloprasum (Egyptian kurrat). *Journal of Veterinary Science and Medical Diagnosis, 4*(4), 1000170-1000173. doi:10.4172/2325-9590.1000170

Food and Agricultural Organization. (2019). Nigeria at a glance. Retrieved April 03, 2019, from http://www.fao.org/nigeria/fao-in-nigeria/nigeria-at-a-glance/en/

Finney, D. J. (1952). *Probit analysis: A statistical treatment of the sigmoid response curve*. Cambridge, United Kingdom: Cambridge University Press.

Gazuwa, S. Y., Makanjuola, E. R., Jaryum, K. H., Kutshik, J. R., & Mafulul, S. G. (2013). The phytochemical composition of *Allium cepa/ Allium sativum* and the effects of their aqueous extracts (cooked and raw forms) on the lipid profile and other hepatic biochemical parameters in female albino Wistar rats. *Asian Journal of Experimental Biological Sciences*, *4*(3), 406-410.

Griffiths, G., Trueman, L., Crowther, T., Thomas, B., & Smith, B. (2002). Onions - A global benefit to health. *Phytotherapy Research, 16*(7), 603-615.

Iloki-Assanga, S. B., Lewis-Luján, L. M., Lara-Espinoza, C. L., Gil-Salido, A. A., Fernandez-Angulo, D., Rubio-Pino, J. L., & Haines, D. D. (2015). Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and *Phoradendron californicum*. *BMC Research Notes*, *8*(1), 396-410. doi: 10.1186/s13104-015-1388-1

Johansen, R., Needham, J. R., Colquhoun, D. J., Poppe, T. T., & Smith, A. J. (2006). Guidelines for health and welfare monitoring of fish used in research. *Laboratory Animals*, *40*(4), 323–340.

Kirk, S. R., Sawyer, R., & Egan, H. (1991). Pearson's composition and analysis of foods (9th ed.). Singapore: Longman Publishers.

Kuete, V. (2017). *Medicinal spices and vegetables from Africa: Therapeutic potential against metabolic, inflammatory, infectious and systemic diseases*. Cambridge, USA: Academic Press.

Manilal, A., Sujith, S., Kiran, G. S., Selvin, J., Shakir, C., Gandhimathi, R., & Lipton, A. P. (2009). Antimicrobial potential and seasonality of red algae collected from the Southwest coast of India tested against shrimp, human and phytopathogens. *Annals of Microbiology*, *59*(2), 207-219.

Marcon, L., Bazzoli, N., Mounteer, A. H., & dos Anjos Benjamin, L. (2015). Histological and histometric evaluation of the liver in *Astyanax Bimaculatus* (Teleostei: Characidae), exposed to different concentrations of an organochlorine insecticide. *Anatomical Record*, *298*(10), 1754–1764. doi: 10.1002/ar.23196

Martini, R., & Lindberg, C. (2013). *Fishing for Tomorrow: Managing fisheries for sustainable development*. Retrieved April 03, 2019, from http://www.oecd.org/gov/pcsd/002013401_Coherence%20for%20Development_Dec_PDFX_WEB.pdf

National Bureau of Statistics. (2019). *Nigerian gross domestic product report: Q4 and full year 2018*. Abuja, Nigeria: NBS.

Nebedum, J., Ajeigbe, K., Nwobodo, E., Uba, C., Adesanya, O., Fadare, O., & Ofusori, D. (2009). Comparative study of the ethanolic extracts of four Nigerian plants against some pathogenic microorganisms. *Research Journal of Medicinal Plants*, *3*(1), 23-28. doi: 10.3923/rjmp.2009.23.28

Nicastro, H. L., Ross, S. A., & Milner, J. A. (2015). Garlic and onions: Their cancer prevention properties. *Cancer Prevention Research*, *8*(3), 181-189. doi: 10.1158/1940-6207.CAPR-14-0172

Obaroh, I. O., Keta, J. N., & Nwaogu, J. (2018). Growth performance of *Clarias gariepinus* fed crude extracts of *Allium cepa*, *Moringa oliefera* and *Vernonia amygdalina*. *Asian Journal of Animal Science*, *12*(1), 36-41. **doi:** 10.3923/ajas.2018.36.41

Obioha, U. E., Suru, S. M., Ola-Mudathir, K. F., & Faremi, T. Y. (2009). Hepatoprotective potentials of onion and garlic extracts on cadmium-induced oxidative damage in rats. *Biological Trace Element Research*, *129*(1-3), 143-156. doi: 10.1007/s12011-008-8276-7

Ogbonna, O. J., Udia, P. M., Abe, P. N., Omoregha, C. U., & Anele, E. I. (2016). Phytochemical and proximate analysis, mineral and vitamin compositions of *Allium cepa* bulb extract. *Advances in Biomedicine and Pharmacy*, *3*(4), 181-186. doi: 10.2016/adbp

Ohikhena, F. U., Wintola, O. A., & Afolayan, A. J. (2016). Toxicity assessment of different solvent extracts of the medicinal plant, *Phragmanthera capitata* (Sprengel) balle on brine shrimp (*Artemia salina*). *International Journal of Pharmacology*, *12*(7), 701-710. **doi:** 10.3923/ijp.2016.701.710.

Ola-Mudathir, K. F., & Maduagwu, E. N. (2014). Antioxidant effects of methanol extract of *Allium cepa* Linn. on cyanide-induced renal toxicity in male Wistar rats. *Nigerian Journal of Physiological Sciences*, *29*(2), 147-151.

Opiyo, M. A., Marijani, E., Muendo, P., Odede, R., Leschen, W., & Charo-Karisa, H. (2018). A review of aquaculture production and health management practices of farmed fish in Kenya. *International Journal of Veterinary Science and Medicine*, *6*(2), 141–148. doi:10.1016/j.ijvsm.2018.07.001

Ozkurt, S., Acikalin, M. F., Temiz, G., Akay, O. M., & Soydan, M. (2014). Renal hemosiderosis and rapidly progressive glomerulonephritis associated with primary hemochromatosis. *Renal Failure*, *36*(5), 814-6. doi: 10.3109/0886022X.2014.892391

Parton, K. (2000). Onion toxicity in farmed animals. *New Zealand Veterinary Journal*, *48*(3), 89-89. doi: 10.1080/00480169.2000.36168

Patil, M. V., & Patil, D. A. (2007). Some herbal remedies used by the tribals of Nasik district, Maharashtra. *Natural Product Radiance*, *6*(2), 152-157.

Pulvertaft, R. J. V. (1950). Museum techniques: A review. *Journal of Clinical Pathology*, *3*(1), 23.

Relia, N., & Kaushik, C (2010). Renal hemosiderosis: A case of black kidneys causing renal failure. *Journal of Postgraduate Medicine*, *56*(3), 216-217. doi: 10.4103/0022-3859.68635.

Salami, H. A, John, A. I., & Ekanem, A. U. (2012). The effect of aqueous preparation of *Allium cepa* (onion) and *Allium sativa* (garlic) on erythrocyte osmotic fragility in wistar rats: *In vivo* and *in vitro* studies. *Nigerian Journal of Physiological Sciences*, *27*(1), 29-34.

Saleh, N. E., Michael, F. R., & Toutou, M. M. (2015). Evaluation of garlic and onion powder as phyto-additives in the diet of sea bass (*Dicentrarcus labrax*). *Egyptian Journal of Aquatic Research*, *41*(2), 211-217.

Santosh, C. H., Attitalla, I. H., & Mohan, M. M. (2013). Phytochemical analysis, antimicrobial and antioxidant activity of ethanolic extract of *Vernonia anthelmintica*. *International Journal of Pharmacy and Biological Sciences*, *4*(1), 960-966.

Syahmi, A. R. M., Vijayarathna, S., Sasidharan, S., Latha, L. Y., Kwan, Y. P., Lau, Y. L., … Chen, Y. (2010). Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* Jacq. (oil palm leaf) methanol extract. *Molecules*, *15*(11), 8111–8121. doi: 10.3390/molecules15118111

Ugwu, E. C., & Omale, J. (2011). Comparative effects of aqueous garlic (*Allium sativum*) andonion (*Allium cepa*) extracts on some haematological and lipid indices of rats. *Annual Review and Research in Biology*, *1*(3), 37-44.

Wani, A. A., Sikdar, M., & Zube, S. M. (2018). Nephroprotective action of taurine and garlic extract against copper sulphate induced renal toxicity in *Clarias gariepinus*. *International Journal of Pharmacy and Biological Sciences*, *8*(1), 2321-3272.

Widyawati, P. S., Budianta, T. D. W., Kusuma, F. A., & Wijaya, E. L. (2014). Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indicia* less leaves extracts. *International Journal of Pharmacognosy and Phytochemical Research*, *6*(4), 850-855

Yarahmadi, P., Miandare, H. K., & Hoseinifar, S. H. (2016). Hemato-immunological and serum biochemical parameters, intestinal histomorphology and growth performance of rainbow trout (*Oncorhynchus mykiss*) fed dietary fermentable fiber (Vitacel®). *Aquaculture Nutrition*, *22*(5), 1134-1142. doi: 10.1111/anu.12388

Yatmark, P., Morales, N. P., Chaisri, U., Wichaiyo, S., Hemstapat, W., Srichairatanakool, S., & Fucharoen, S. (2016). Iron distribution and histopathological study of the effects of deferoxamine and deferiprone in the kidneys of iron overloaded β-thalassemic mice. *Experimental and Toxicologic Pathology*, *68*(8), 427-34. doi: 10.1016/j.etp.2016.06.006

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Biofilm Formation and Survival of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* on Tomato Phylloplane

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ABSTRACT

Various human enteric pathogens have been isolated from surface of spinach, lettuce, sprouts, tomato, radish, berries etc. These microbes are not endemic to plant surface but they adapt and survive by mechanism(s) which are still unknown. This study was aimed to understand the colonization pattern of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* by leaf impression method on tomato plants raised under aseptic conditions. The biofilm forming ability of these bacteria were also studied. The study revealed that the population of these enteric pathogens were significantly high (89 CFU/cm^2) on phylloplane of tomato after 96 hours of incubation. Each of these microbes had a distinct colonization pattern and could successfully form biofilm. The study throws light on the ability of human enteric pathogens to colonize phylloplane possibly aided by their biofilm forming capability on leaf surface. The study is significant since it shall enhance understanding of association of human enteric pathogens with plants to design strategies for their survival.

Keywords: Biofilm, *Chryseobacterium jejuense*, colonization, cross talk, human enteric pathogen, *Klebsiella pneumoniae*, *Serratia fonticola*

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INTRODUCTION

Increasing number of food borne gastrointestinal outbreaks is a global concern. Consumption of fresh produce as a part of organic and healthy diet has been reported to be associated with gastrointestinal disorders (Brandl & Mandrell, 2002; Martínez-Vaz et al., 2014). Important sources of human enteric pathogens are irrigation water and untreated organic manure in pre and post-harvest environment. *Escherchia coli* O157:H7 and *Salmonella enterica* have been reported to contaminate leafy greens (Bolton et al., 2011; Buchholz et al., 2012; Talley et al., 2009; Wachtel & Charkowski, 2002; Wasala et al., 2009). Other human pathogenic bacteria isolated from surface of tomato and radish include *Serratia fonticola*, *Klebsiella pneumoniae*, *Enterobacter ludwigi*, *Chryseobacterium jejuense* and *Stenotrophomonas maltophilia* (Gaur et al., 2016). A number of environmental factors affect the colonization and internalization of these bacteria on phylloplane. These factors include poor nutrient availability and aerobic conditions, exposure to ultra violet radiations. Further, presence of curli and fimbriae on bacteria facilitates their adhesion on the leaf surface (Brandl et al., 2005; Dinu & Bach, 2011; Erickson et al., 2010; Linden et al., 2013).

Bacteria which enter the plant system communicate and exhibit a density dependent behaviour. This environmental sensing system, which is used by bacteria to monitor their growth, is called quorum sensing (Fuqua et al., 1994). This system is established through small signaling molecules called autoinducers (Brelles-Mariño & Bedmar, 2001; Gowda et al., 2013; Holden et al., 2002). Gram negative bacteria secrete acylated homoserine lactone molecules (Waters & Bassler, 2005) and Gram positive bacteria secrete cyclic peptides as quorum sensing signals (Kleerebezem et al., 1997).

In the present study, bacteria isolated from phylloplane of tomato namely *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* have been chosen for study of colonization and biofilm forming capabilities. These bacteria have been reported to be potent human pathogens and cause severe diseases in immune-compromised individuals (Aljorayid et al., 2016; Caprioli et al., 2005; Gavini et al., 1979; Lopes et al., 2005). These bacteria have also been found to be resistant against broad range of antibiotics.

Although studies for Gram negative quorum sensing have been reported from plant associated bacteria, but the ability of human enteric pathogens to form biofilms on plant surface is not well elucidated. Tomato being the largest cultivated vegetable crop is widely consumed as fresh salad has been used in this study. The study is aimed to understand the biofilm forming capability of the selected human enteric pathogens which were found to be colonizing aerial parts of plants. Biofilms help microbes to adapt and survive on the substratum they colonize. The study of biofilm is important because effective formation of biofilm by a microbe ensures its survival under various conditions. In the present study, understanding the biofilm formation would help to unravel the intrinsic underlined aspects of its colonization on plant surface. The results would help us to device ways and means to disrupt the biofilm formation thereby making fresh farm produce safe for human consumption. The study would help to identify the capacity of these microbes so that the colonization potential of these formidable human pathogens could be drastically reduced by disrupting their biofilm forming capacity or biofilm formation. This would enhance the safety of food products for human consumption. The procedure is unique as it provides a direct insight into the biofilm formation by human enteric pathogens on plants.

MATERIALS AND METHODS

Plant Material

Seeds of *Solanum lycoperscicum* (var. Pusa Ruby) were procured from National Seed Corporation, New Delhi, India. Seeds were surface sterilized using 0.1% Sodium hypochlorite solution followed by washing with sterilized distilled water to remove traces of hypochlorite. Seeds were sown in sterilized soilrite in plastic trays (35cm × 25 cm × 6cm; L × W × H). Plants were grown at 25±1ºC and 70% relative humidity with 12 hour (L/D) photoperiod under aseptic conditions. Plants were watered daily with sterile distilled water and weekly with sterilized 100% Hoagland's solution.

Bacterial Cultures

Inoculum of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* were prepared from glycerol stocks maintained at -20ºC. These bacteria were isolated from the fruits and leaves surface of field grown tomato, which used organic manure and underground water for irrigation. Fifty mL of sterilized Nutrient broth was inoculated with 1 mL each of the selected pathogens and incubated overnight at 37±1ºC on an orbital shaker incubator. The inocula of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* were prepared from this overnight stock of cultures by adjusting their concentration to 10^8cells/mL (optical density of 0.1 at 600nm), according to the method described by Cottenye (2010). Combination of bacterial cultures was prepared by mixing respective cultures (1:1) and 0.1 OD maintained at 600 nm (10^8 cells/mL).

Treatment and Sampling of Plants

Eight-weeks-old plants were chosen for the study. Plants were divided into seven groups. Experiment was conducted with three replicates. Each replicate had 25 plants:

Group 1- inoculated with *Serratia fonticola*

Group 2 - inoculated with *Klebsiella pneumoniae*

Group 3 - inoculated with *Chryseobacterium jejuense*

Group 4- inoculated with combination of *Serratia fonticola* and *Klebsiella pneumoniae*

Group 5 - inoculated with combination of *Serratia fonticola* and *Chryseobacterium jejuense*

Group 6 - inoculated with combination of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense*

Group 7 - sprayed with sterile distilled water (control).

The plants of each group were inoculated with 50 mL suspension of the bacteria using sterile atomizer. The treatment was carried out under aseptic condition.

Colonization Assay

The third node leaf from both control and treated plants were sampled at 0, 24, 48, 72 and 96 hours post inoculation (hpi) and placed in sterile polythene bags. Five leaf samples from each replicate were collected and colonization pattern was studied by leaf impression techniques as described by Aneja (2003). Nutrient agar media was prepared, sterilized and plated in petri dishes (9 cm diameter).

The adaxial and abaxial surfaces of the sampled leaves were pressed on surface of media. The leaves were subsequently removed. The plates were incubated at 37±1ºC for overnight in an orbital shaker incubator. The process was repeated for each sample of each replicate for sampling intervals of each group. Colonies were counted and results were expressed as CFU/ cm^2 of leaf. Statistical test for significance was carried out by GraphPad software. Number of CFU observed were analysed for statistical significance using t- test. Average of triplicates was calculated followed by standard error. The two tailed P values were calculated at 95% level of confidence.

Quantification of Biofilm Formation

Biofilm formation was determined and quantified by microtiter plate method (Boddey et al., 2006). Bacterial cultures grown overnight at 37±1°C with shaking were used for the estimation. 1 mL of nutrient broth media was added into the wells of 96-well plate and 10µL of cultures of *S. fonticola*, *K. pneumoniae* and *C. jejuense* respectively were added singly and in combination into the respective wells containing media. The plate was incubated at 37±1°C for 18 hours. After incubation, a fresh polystyrene plate was taken and 1 mL media was added. Ten (10)µL of respective bacteria from each well was transferred into the triplicate wells of the fresh plate containing media and incubated at 37°C for 24 hours without shaking. Wells containing only media served as control. After 24 hours the supernatant from the wells was carefully removed and 1% crystal violet was added to stain the wells and kept at room temperature for 30 minutes. Then stain was removed and wells were washed carefully twice with 175µL of deionized water followed by addition of 175µL dimethyl sulfoxide (DMSO) to solubilize the crystal violet. Then absorbance was recorded spectrophotometrically using ELISA reader at a wavelength of 570 nm. The assay was run separately for each strain in independent experiments.

RESULTS AND DISCUSSION

Colonization Assay

The study revealed that each bacterial species and their combinations had a unique colonization pattern on tomato leaves. No morphological or pathological changes were observed on the plants (Figure 1). The findings of the study strongly suggest that human enteric pathogens are able to effectively colonize plant parts and thereby significantly increase the potential entry into humans through consumption of such contaminated fresh farm produce. The abaxial surface of leaf was more populated than adaxial surface. The colonies were concentrated around the midrib and veins of leaf (Figure 2). CFU count of *Serratia fonticola* was maximum at 24 hour post inoculation which gradually decreased with time and was found to be minimum at 96 hour post inoculation (Figure 3a). The CFU count at 24 hours was 91 CFU per cm^2

Figure 1. Plants inoculated with selected human pathogens. No pathological changes were observed on the plants

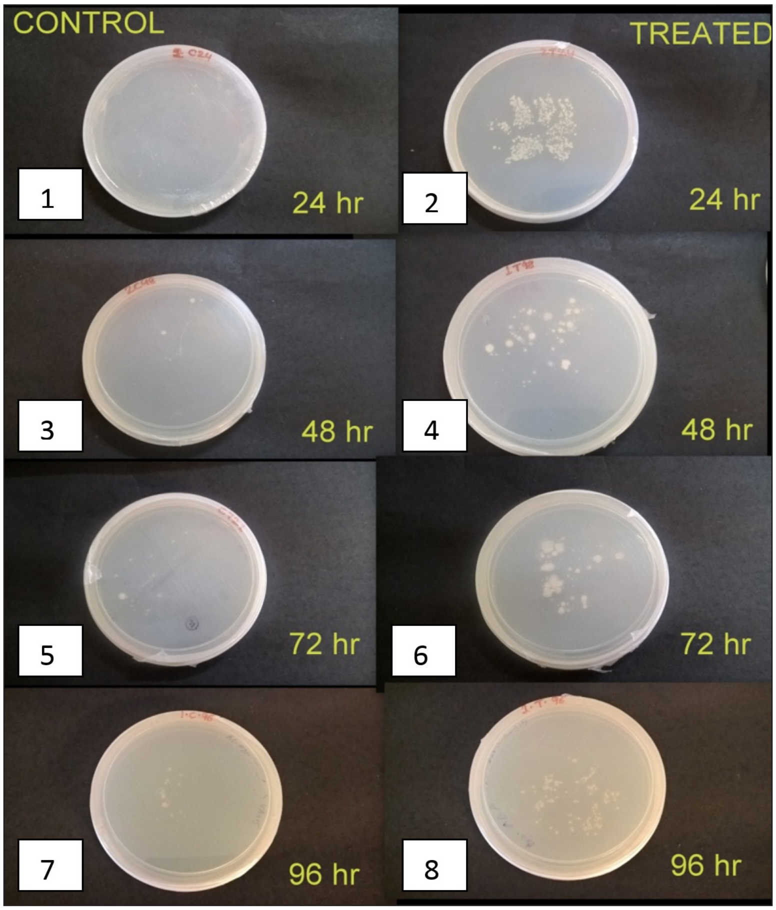


Figure 2. Bacterial colonization by leaf impression technique

($p \leq 0.0001$), which decreased significantly (46 CFU per cm^2) at 96 hours ($p \leq 0.0001$). However in *Klebsiella pneumonia*e and *Chryseobacterium jejuense*, CFU count gradually increased throughout the sampling period (Figures 3b and 3c). In *K. pneumoniae*, CFU count was 56 CFU per cm^2 at 24 hpi ($p \leq 0.0001$) and increased to 89 CFU per cm^2 at 96 hpi ($p \leq 0.0001$). For *C. jejuense*, it was found to be 42 CFU per cm^2 at 24 hpi ($p \leq 0.0001$) which increased to 60 CFU per cm^2 at 96 hpi ($p \leq 0.0001$). Inoculation of mixed bacterial colonies resulted in similar colonization pattern. Combination of *S. fonticola* and *C. jejuense* as well as that of *S. fonticola*, *K. pneumoniae* and *C. jejuense* showed similar pattern (Figures 3e and 3f). In both the combinations, maximum CFU was recorded after 96 hours post inoculation. However, the combination of *S. fonticola* and *K. pneumoniae* had a different pattern of colonization. (Figure 3d). CFU count initially increased during 24 to 48 hr sampling and then gradually decreased (72 to 96 hours post inoculation). The count was 50 CFU ($p \leq 0.0001$) and 103 CFU per cm^2 at 24 and 48 hpi respectively ($p \leq 0.0001$) and then decreased to 66 CFU and 61 CFU per cm^2 at 72 and 96 hpi ($p \leq 0.0001$) respectively. Bacterial inoculation either singly or in combination had varying results. More number of colonies were located on abaxial surface than on adaxial surface. Most of the colonies were around midrib and veins. It supports the fact that abaxial surface has more stomata which are the sites of leakage and accumulation of nutrients. Microbes generally tend to colonize where nutrients are available. Leben (1988) had also reported similar observations. Results suggest that bacteria are able to successfully colonize and significantly multiply on the phylloplane. It suggests that these human enteric pathogens have mechanisms to successfully adapt and colonize plant surfaces. Though these bacteria were isolated from the same niche, but all of them were found to have varying adaptation characteristics, when inoculated either single and in combination. The protocol followed for study is unique because it strictly helps in isolation and enumeration of human enteric pathogens from plant surface under aseptic conditions.

The findings are unique because the microbes are evolutionarily adapted to colonise human gut, but are able to successfully colonise, multiply and survive on phylloplane. The study highlights the possibility of HEPs entering human system through consumption of raw fruits and vegetables and thereby becoming major health hazard. *E. coli* O157:H7 has been reported on Alfalfa which persisted for long period (Cools et al., 2001; Gagliardi & Karns, 2002) but survival of *S. fonticola*, *K. pneumoniae* and *C. jejuense* on plant surfaces have not been studied much. The colonization of various enteric bacteria under field conditions is governed by numerous environmental factors like nutrient availability, fluctuation of temperature and humidity (Beattie & Lindow, 1994; Suslow, 2002). Short term changes in the weather conditions at the microscale level of leaves were earlier thought to be the reason for variation in population size of various bacteria on phylloplane.

Therefore modifications and optimum growth conditions on specific sites on leaves allow microbes to persist on plant for long time (Hirano & Upper, 2000). Similarly, in field grown parsley, the inoculum of *E. coli* and *S. enterica*, could colonize the plant surface for up to 177 and 231 days respectively (Islam et al., 2004). In lettuce

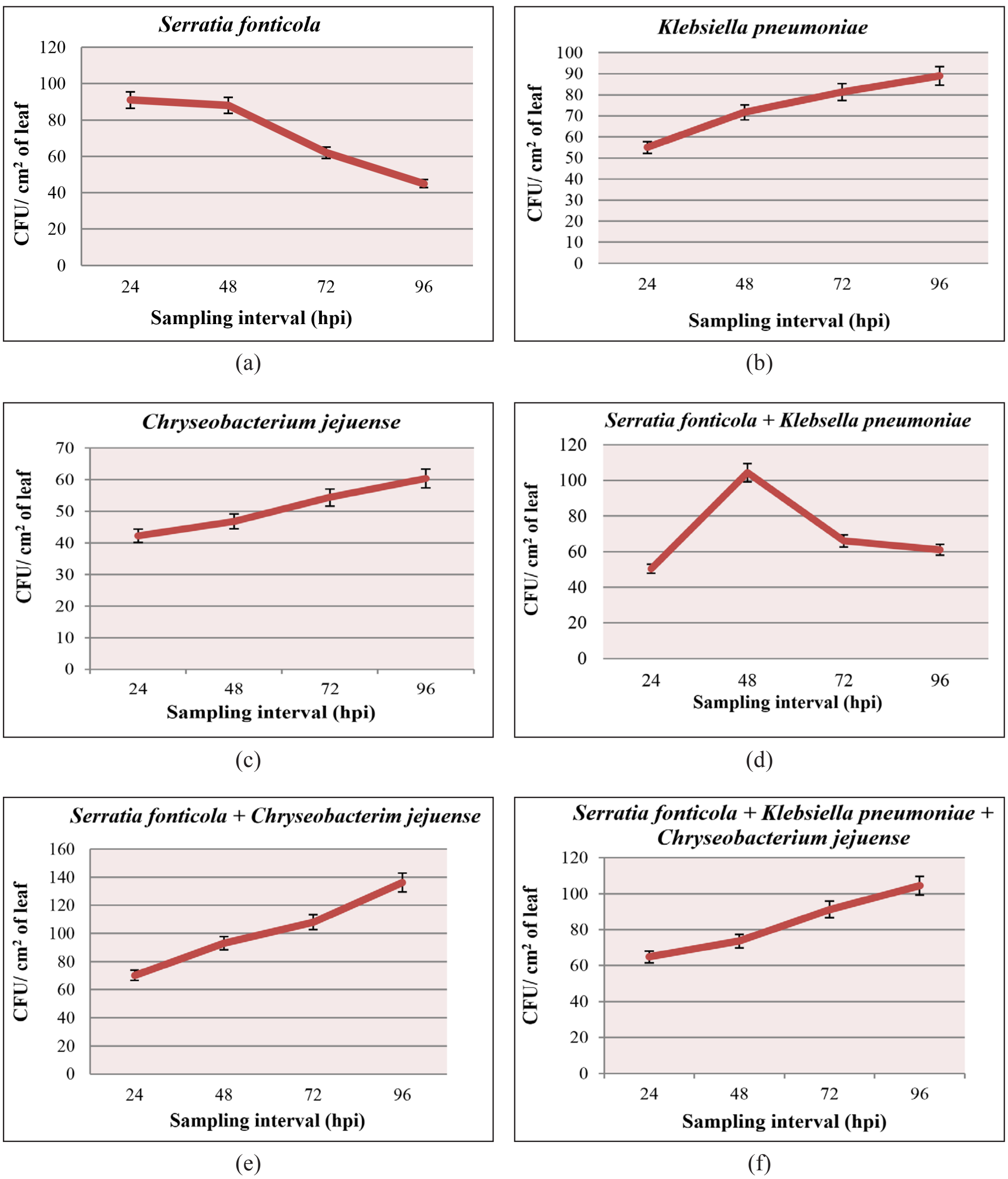


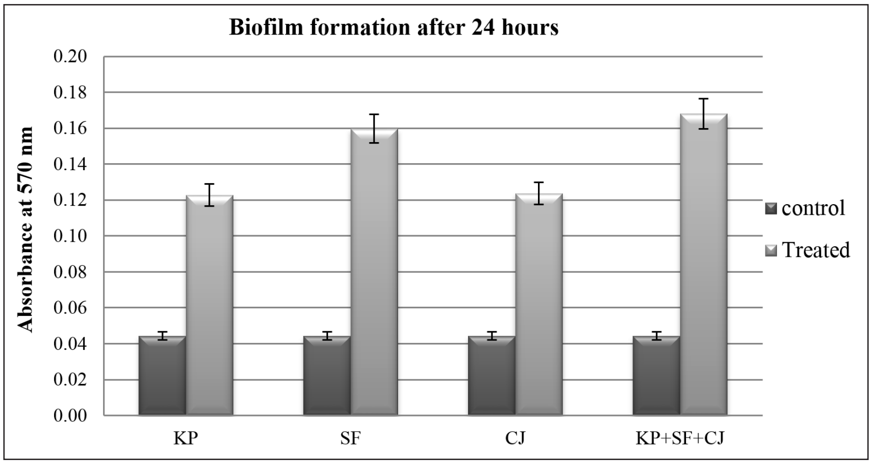
Figure 3. CFU count of (a) *Serratia fonticola*; (b) *Klebsiella pneumoniae;* (c) *Chryseobacterium jejuense;* (d) *Serratia fonticola* and *Klebsiella pneumoniae;* (e) *Serratia fonticola* and *Chryseobacterium jejuense; and* (f) *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense*
Vertical bars represent standard error

leaves the colonization and persistence of enteric pathogens was reported for upto 30 days (Solomon et al., 2003).

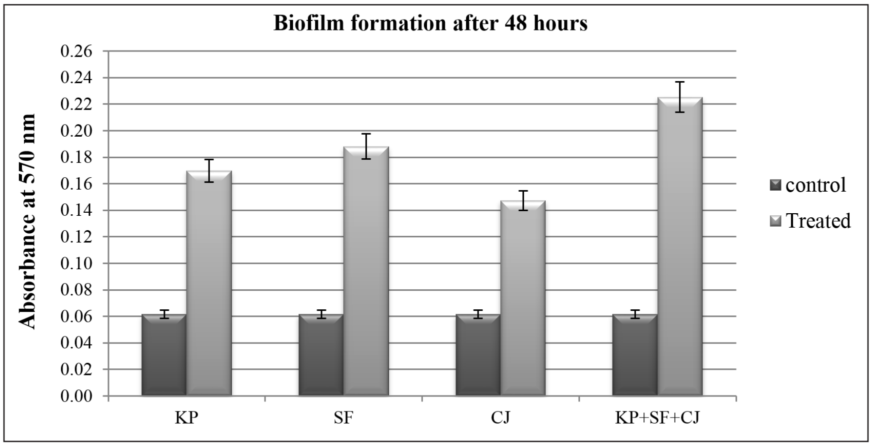
These human enteric pathogens modify the environment of the leaf surface, thereby making it suitable for their own colonization (Kwan et al., 2013; Potnis et al., 2015). The resident bacteria which are natural colonizers of phylloplane also influence the colonization of phylloplane by HEPs (Pollard et al., 2014; Poza-Carrion et al., 2013). Colonization of enteric pathogens is also regulated by expression of a number of genes. Barak et al. (2007) had reported that *Salmonella enterica* mutant which lacked bcsA gene, which was responsible for cellulose synthase production had a poor colonization than wild type strains. This is possibly because cellulose is one of the components for formation of biofilms. Any drop in biofilm formation would impact the colonization potential of the microbe. Lim et al. (2014) and Roy et al. (2013) had reported that differential gene expression was exhibited by different bacteria, in order to colonize plant surface. Hence in the present study, colonization by the pathogenic bacteria at certain specific sites is supported by the fact that these bacteria can modify the local environment so that they can persist and replicate on the phyllosphere. Gilbert et al. (2003) had proposed that microbial population of $<10^3$ on any edible plant surface were considered to be within the safe limits. According to this parameter, pathogens in present study colonize and persist on tomato phylloplane beyond the threshold concentration.

Quantification of Biofilm Formation

Colonization has been further studied by the ability of these human enteric pathogens to form biofilms. Bacteria which colonize on leaf surface, establish cell to cell communication through quorum sensing, which leads to aggressive growth of bacterial cells even after 96 hours post inoculation. Quantification of biofilm formation demonstrated that all the three bacterial species used in the present study could significantly form biofilm. Among the three bacterial isolates, *Serratia fonticola* had a significantly higher biofilm forming capacity as compared to *Klebsiella pneumoniae* and *Chryseobacterium jejuense*. The combination of *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* exhibited highest biofilm formation capacity as compared to any individual species. Absorbance recorded for *S. fonticola* after 24 hours of incubation was 0.159 ($p \leq 0.0001$), while those for *K. pneumoniae* and *C. jejuense* was 0.122 ($p \leq 0.0001$) and 0.123 ($p \leq 0.0001$) respectively. However absorbance recorded for combination of three bacteria was 0.168 ($p \leq 0.0001$). Biofilm formation was found to be highest after 48 hours of incubation as compared to 24 hours (Figure 4a). Absorbance recorded for *S. fonticola* after 24 hours of incubation was 0.188 ($p \leq 0.0001$), while those for *K. pneumoniae* and *C. jejuense* was 0.169 ($p \leq 0.0001$) and 0.147 ($p \leq 0.0001$) respectively. After 48 hours, *Serratia fonticola* could significantly form biofilm as compared to the other two bacteria (Figure 4b). Absorbance recorded for combination of



(b)



(a)

Figure 4. Biofilm formation by bacterial isolates (a) after 24 hours; and (b) after 48 hours
Vertical bars represent standard error
Note:. KP = *Klebsiella pneumoniae*; SF = *Serratia fonticola*; CJ = *Chryseobacterium jejuense*; KP+SF+CJ = Combination of *Klebsiella pneumoniae*, *Serratia fonticola* and *Chryseobacterium jejuense*]

three bacteria was 0.225($p \leq 0.0001$), which was found to be highest among all. Results were found to be statistically significant.

Biofilms have been found to be associated with several molecules like acyl homoserine lactones, oligopeptides and amino acids (Loehfelm et al., 2007; McLean et al., 1997). Gram negative bacteria are able to produce acyl homoserine lactones which have been reported to control several genes for colonization (Costerton et al., 1999). Brandl and Mandrell (2002) had reported that bacteria formed biofilm or aggregates. Microbial biofilms can be formed on leaves roots, intercellular spaces of plant tissues. The microbes form biofilms to prevent themselves from desiccation, environmental stress, antimicrobial compounds etc. On the phyllosphere, biofilms impact the epiphytic fitness of the microbes (Costerton et al., 1987; Morris et al., 1997; Zottola & Sasahara, 1994). It has been reported that biofilm help in their stability and persistence on phylloplane making them fit to survive. The studies reported so far, do not characterize the quorum sensing molecules produced by these human enteric pathogens on plant surface through the biofilm forming capacity exhibited by these human pathogenic bacteria is of a concern for the society as it possibly enhances their adaptability on plants and thereby enhance their survival in an otherwise harsh environment on leaf surface.

Niemira and Cooke (2010) reported that some human pathogenic bacteria were capable of modulating gene expression involved in biofilm formation. Certain biofilm forming components like curli and cellulose are involved in the quorum sensing. Bacteria produce signal molecules which diffuse from cells and get accumulated in the extracellular environment. Upon reaching a threshold concentration, these signals regulate the expression of certain genes for LuxR/LuxI, in turn regulating their transcription (Fuqua et al., 1996; Gray, 1997; Hardman et al., 1998).

In the present study, all three human pathogenic bacteria namely *Serratia fonticola*, *Klebsiella pneumoniae* and *Chryseobacterium jejuense* have been found to form microbial biofilms. Identification of these molecules would help in limiting the bacterial colonization on phylloplane. Quorum sensing mechanisms of these bacteria are involved in antibiotic resistance against a broad spectrum of antibiotics and elimination of competitors from same niche (Brelles-Mariño & Bedmar, 2001). Pathogens like *Pseudomonas aeruginosa* have been studied for quorum sensing mechanism and virulence factors have been isolated and studied (Anzai et al., 2000).

The study of such factors in plant system would lead to limit the pathogen load on fresh produce. Developing quorum sensing inhibitors can prove to be a rational approach towards food safety.

CONCLUSION

The study revealed that human enteric pathogens namely, *Serratia fonticola*, *Klebsiella pneumoiae* and *Chryseobacterium jejuense* were able to form biofilm on the phylloplane of tomato. This possibly adapts them to survive and establish on the phylloplane and cause severe disorders.

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REFERENCES

Aljorayid, A., Viau, R., Castellino, L., & Jump, R. L. (2016). *Serratia fonticola*, pathogen or bystander? A case series and review of the literature. *IDCases*, *5*, 6-8. doi: 10.1016/j.idcr.2016.05.003

Aneja, K., R. (2003). Isolation of microorganisms from phyllosphere (Phylloplane). In *Experimental microbiology, plant pathology and biotechnology* (pp. 176-178). New Delhi, India: New Age International Publishers.

Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H., & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology*, *50*(4), 1563-1589. doi: 10.1099/00207713-50-4-1563

Barak, J. D., Jahn, C. E., Gibson, D. L., & Charkowski, A. O. (2007). The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. *Molecular Plant-Microbe Interaction*, *20*(9), 1083-1091. doi: 10.1094/MPMI-20-9-1083

Beattie, G. A., & Lindow, S. E. (1994). Epiphytic fitness of phytopathogenic bacteria: Physiological adaptations for growth and survival. In *Bacterial pathogenesis of plants and animals* (pp. 1-27). Heidelberg, Germany: Springer.

Boddey, J. A., Flegg, C. P., Day, C. J., Beacham, I. R., & Peak, I. R. (2006). Temperature-regulated microcolony formation by *Burkholderia pseudomallei* requires pila and enhances association with cultured human cells. *Infection and Immunity*, *74*(9), 5374-5381. doi: 10.1128/iai.00569-06

Bolton, D., Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., & Mcdowell, D. (2011). Incidence and survival of non-O157 verocytotoxigenic *Escherichia coli* in soil. *Journal of Applied Microbiology*, *111*(2), 484-490. doi: 10.1111/j.1365-2672.2011.05057.x

Brandl, M. T., & Mandrell, R. E. (2002). Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Applied and Environmental Microbiology*, *68*(7), 3614-3621. doi: 10.1128/aem.68.7.3614-3621.2002

Brandl, M. T., Rosenthal, B. M., Haxo, A. F., & Berk, S. G. (2005). Enhanced survival of *Salmonella enterica* in vesicles released by a soilborne *Tetrahymena* species. *Applied and Environmental Microbiology*, *71*(3), 1562-1569. doi: 10.1128/aem.71.3.1562-1569.2005

Brelles-Mariño, G., & Bedmar, E. J. (2001). Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *Journal of Biotechnology*, *91*(2-3), 197-209. doi: 10.1016/s0168-1656(01)00330-3

Buchholz, A. L., Davidson, G. R., Marks, B. P., Todd, E. C., & Ryser, E. T. (2012). Transfer of *Escherichia coli* O157:H7 from equipment surfaces to fresh-cut leafy greens during processing in a model pilot-plant production line with sanitizer-free water. *Journal of Food Protection*, *75*(11), 1920-1929. doi: 10.4315/0362-028x.jfp-11-558

Caprioli, A., Morabito, S., Brugère, H., & Oswald, E. (2005). Enterohaemorrhagic *Escherichia coli*: Emerging issues on virulence and modes of transmission. *Veterinary Research*, *36*(3), 289-311. doi: 10.1051/vetres:2005002

Cools, D., Merckx, R., Vlassak, K., & Verhaegen, J. (2001). Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. *Applied Soil Ecology*, *17*(1), 53-62. doi: 10.1016/s0929-1393(00)00133-5

Costerton, J. W., Cheng, K. J., Geesy, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annual Review of Microbiology*, *41*(1), 435-464. doi: 10.1146/annurev.mi.41.100187.002251

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science*, *284*(5418), 1318-1322. doi: 10.1126/science.284.5418.1318

Cottenye, N. (2010). *Antimicrobial surfaces based on self-assembled nanoreactors: From block copolymer synthesis to bacterial adhesion studies*. Retrieved December 6, 2018, from https://tel.archives-ouvertes.fr/tel-00598560/document

Dinu, L. D., & Bach, S. (2011). Induction of viable but nonculturable *Escherichia coli* O157:H7 in the phyllosphere of lettuce: A food safety risk factor. *Applied and Environmental*

Microbiology, *77*(23), 8295-8302. doi: 10.1128/aem.05020-11

Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., . . . Doyle, M. P. (2010). Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. *Journal of Food Protection*, *73*(6), 1023-1029. doi: 10.4315/0362-028x-73.6.1023

Fuqua, W. C., Winans, S. C., & Greenberg, E. P. (1994). Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology*, *176*(2), 269-275. doi: 10.1128/jb.176.2.269-275.1994

Fuqua, W. C., Winans, S. C., & Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annual Review of Microbiology*, *50*(1), 727-751. doi: 10.1146/annurev.micro.50.1.727

Gagliardi, J., & Karns, S. J. (2002). Persistence of *Escherichia coli* O157:H7 in Soil and on plant roots. *Environmental Microbiology*, *4*(2), 89-96. doi: 10.1046/j.1462-2920.2002.00273.x.

Gaur, I., Sharma, P. D., & Paul, P. K. (2016). Human pathogenic bacteria associated with field grown Tomato and Radish. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, *18*(3), 753- 761.

Gavini, F., Ferragut, C., Izard, D., Trinel, P. A., Leclerc, H., Lefebvre, B., & Mossel, D. A. (1979). *Serratia fonticola*, a new species from water. *International Journal of Systematic Bacteriology*, *29*(2), 92-101. doi:10.1099/00207713-29-2-92

Gilbert, P., McBain, A. J., & Rickard, A. H. (2003). Formation of microbial biofilm in hygienic situations: A problem of control. *International Biodeterioration and Biodegradation*, *51*(4), 245-248. doi: 10.1016/S0964-8305(03)00043-X

Gowda, K. L., John, J., Marie, M. A., Sangeetha, G., & Bindurani, S. R. (2013). Isolation and characterization of quorum-sensing signalling molecules in *Pseudomonas aeruginosa* isolates recovered from nosocomial infections. *Journal of Pathology, Microbiology and Immunology*, *121*(9), 886-889. doi: 10.1111/apm.12047

Gray, K. M. (1997). Intercellular communication and group behavior in bacteria. *Trends in Microbiology*, *5*(5), 184-188. doi: 10.1016/s0966-842x(97)01002-0

Hardman, A. M., Stewart, G. S. A. B., & Williams, P. (1998). Quorum sensing and the cell- cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria. *Antonie Van Leeuwenhoek*, *74*(4), 199-210. doi: 10.1023/A:1001178702503

Hirano, S. S., & Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* — A pathogen, Ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews*, *64*(3), 624-653. doi: 10.1128/MMBR.64.3.624-653.2000

Holden, M. T., Chhabra, S. R., Nys, R. D., Stead, P., Bainton, N. J., Hill, P. J., . . . Williams, P. (2002). Quorum-sensing cross talk: Isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Molecular Microbiology*, *33*(6), 1254-1266. doi: 10.1046/j.1365-2958.1999.01577.x

Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Persistence of enterohemorrhagic *Escherichia coli* O157: H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection*, *67*(7), 1365-1370. doi: 10.4315/0362-028X-67.7.1365

Kleerebezem, M., Quadri, L. E., Kuipers, O. P., & Vos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Molecular Microbiology*, *24*(5), 895-904. doi:10.1046/j.1365-2958.1997.4251782.x

Kwan, G., Charkowski, A. O., & Barak, J. D. (2013). *Salmonella enterica* suppresses *Pectobacterium carotovorum* subsp. *carotovorum* population and soft rot progression by acidifying the microaerophilic environment. *MBio*, *4*(1), e00557-12. doi:10.1128/mbio.00557-12

Leben, C. (1988). Relative humidity and the survival of epiphytic bacteria with buds and leaves of cucumber plants. *Phytopathology*, *78*(2), 179-185. doi: 10.1094/phyto-78-179

Lim, J., Lee, D. H., & Heu, S. (2014). The interaction of human enteric pathogens with plants. *The Plant Pathology Journal*, *30*(2), 109-116. doi: 10.5423/ppj.rw.04.2014.0036

Linden, I. V., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., & Heyndrickx, M. (2013). Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butter head lettuce seeds, and their subsequent survival and growth on the seedlings. *International Journal of Food Microbiology*, *161*(3), 214-219. doi: 10.1016/j.ijfoodmicro.2012.12.015

Loehfelm, T. W., Luke, N. R., & Campagnari, A. A. (2007). Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *Journal of Bacteriology*, *190*(3), 1036-1044. doi: 10.1128/jb.01416-07

Lopes, A. C., Rodrigues, J. F., & Júnior, M. A. (2005). Molecular typing of *Klebsiella pneumoniae* isolates from public hospitals in Recife, Brazil. *Microbiological Research*, *160*(1), 37-46. doi: 10.1016/j.micres.2004.09.007

Martínez-Vaz, B. M., Fink, R. C., Diez-Gonzalez, F., & Sadowsky, M. J. (2014). Enteric pathogen-plant interactions: molecular connections leading to colonization and growth and implications for food safety. *Microbes and Environments*, *29*(2), 123-135. doi: 10.1264/jsme2.me13139

McLean, R. J. C., Whiteley, M., Stickler, D. J., & Fuqua, W. C. (1997). Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiology Letters*, *154*(2), 259-263. doi: 10.1016/s0378-1097(97)00336-4

Morris, C. E., Monier, J., & Jacques, M. (1997). Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. *Applied and Environmental Microbiology*, *63*(4), 1570-6.

Niemira, B. A., & Cooke, P. H. (2010). *Escherichia coli* O157:H7 biofilm formation on romaine lettuce and spinach leaf surfaces reduces efficacy of irradiation and sodium hypochlorite washes. *Journal of Food Science*, *75*(5), M270-M277. doi: 10.1111/j.1750-3841.2010.01650.x

Pollard, S., Barak, J., Boyer, R., Reiter, M., Gu, G., & Rideout, S. (2014). Potential interactions between *Salmonella enterica* and *Ralstonia solanacearum* in tomato plants. *Journal of Food Protection*, *77*(2), 320-324. doi: 10.4315/0362-028x.jfp-13-209

Potnis, N., Colee, J., Jones, J. B., & Barak, J. D. (2015). Plant pathogen-induced water-soaking promotes *Salmonella enterica* growth on tomato leaves. *Applied and Environmental Microbiology*, *81*(23), 8126-8134. doi: 10.1128/aem.01926-15

Poza-Carrion, C., Suslow, T., & Lindow, S. (2013). Resident bacteria on leaves enhance survival of immigrant cells of *Salmonella enterica*. *Phytopathology*, *103*(4), 341-351. doi: 10.1094/phyto-09-12-0221-fi

Roy, D., Panchal, S., Rosa, B. A., & Melotto, M. (2013). *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica Typhimurium* SL1344. *Phytopathology, 103*(4), 326-332. doi: 10.1094/phyto-09-12-0230-fi

Solomon, E. B., Pang, H. J., & Matthews, K. R. (2003). Persistence of *Escherichia coli* O157:H7 on lettuce plants following spray irrigation with contaminated water. *Journal of Food Protection*, *66*(12), 2198-2202. doi: 10.4315/0362-028X-66.12.2198

Suslow, T. (2002). Production practices affecting the potential for persistent contamination of plants by microbial food pathogens. In S. E. Lindow, E. I. Hecht-Poinar, & V. J. Elliott (Eds.), *Phyllosphere microbiology* (pp 241– 256). St. Paul: USA: APS Press.

Talley, J. L., Wayadande, A. C., Wasala, L. P., Gerry, A. C., Fletcher, J., Desilva, U., & Gilliland, S. E. (2009). Association of *Escherichia coli* O157:H7 with filth flies (Muscidae and Calliphoridae) captured in leafy greens fields and experimental transmission of *E. coli* O157:H7 to spinach leaves by house flies (Diptera: Muscidae). *Journal of Food Protection*, *72*(7), 1547-1552. doi: 10.4315/0362-028x-72.7.1547

Wachtel, M. R., & Charkowski, A. O. (2002). Cross-contamination of lettuce with *Escherichia coli* O157:H7. *Journal of Food Protection*, *65*(3), 465-470. doi: 10.4315/0362-028x-65.3.465

Wasala, L., Talley, J. L., Desilva, U., Fletcher, J., & Wayadande, A. (2013). Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). *Phytopathology*, *103*(4), 373-380. doi: 10.1094/phyto-09-12-0217-fi

Waters, C. M., & Bassler, B. L. (2005). Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology*, *21*(1), 319-346. doi:10.1146/annurev.cellbio.21.012704.131001

Zottola, E. A., & Sasahara, K. C. (1994). Microbial biofilms in the food processing industry - Should they be a concern?. *International Journal of Food Microbiology*, *23*(2), 125-148. doi: 10.1016/0168-1605(94)90047-7

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Isolation and Optimisation of Polylactic Acid (PLA)-packaging-degrading Actinomycete for PLA-packaging Degradation

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ABSTRACT

Nowadays polylactic acid (PLA) is used extensively with respect to environmental concern and good practice in solid-waste management, as food packaging. However, the study on biodegradation of PLA-packaging is very limited. In this work, isolation, identification and optimisation of biomass production of PLA-packaging-degrading actinomycete were carried out. The isolate KKU215 gave the highest cell density in basal medium containing 1.0 g/L PLA-packaging as sole carbon source after 4-week incubation. The weight loss of PLA-packaging degraded by the isolate was 2.65%. The results from scanning electron microscopy analysis indicated that the isolate KKU215 could clearly degrade PLA-packaging. The degradation of pure PLA by KKU215 as clear zone formation on emulsified PLA agar plate was observed. The 16S rDNA gene sequence of the isolate KKU215 was related to the genus *Streptomyces*. Thus, the isolate KKU215 was assigned as *Streptomyces* sp. KKU215. Concentration of yeast extract, initial pH value, temperature and agitation speed for PLA-packaging degradation were optimised by response surface methodology with Box-Behnken design. The highest growth of *Streptomyces* sp. KKU215 was found in basal medium with 3.26% yeast extract, initial pH value of 7.69, and agitated at 149 rpm at 33.7°C. Streptomyces sp. KKU215 when cultured in the optimal conditions could enhance PLA-packaging weight loss from 2.65% to 84.04%, which was 81.39% higher than the unoptimised conditions. Up to date, there is no report of *Streptomyces* species, similar with *Streptomyces* sp. KKU215 capable of degrading PLA. Therefore, *Streptomyces* sp. KKU215, a

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novel polylactic acid-packaging-degrading actinomycete is a promising strain for use in biodegradation of PLA-packaging.

Keywords: Biodegradation, bioplastic, Box-Behnken design, optimisation, polylactic acid (PLA)-packaging, response surface methodology (RSM), *Streptomyces* sp. KKU215

INTRODUCTION

Synthetic plastics are considered as non-degradable materials that generate pollution. The pollution from synthetic plastics derived from petroleum has reached 300 million tons (Thompson et al., 2009) and continues to grow therefore posing a global environmental problem. Biodegradable plastics were used as substitutes for synthetic plastics in the reduction of pollutants or waste. Numerous research are concentrated on the development of novel bioplastics that are susceptible to degradation in the environment. Bioplastics are biodegradable polymers derived from renewable resources such as alginic acid, cellulose and cellulose acetate, chitin and chitosan, gellan gum, hyaluronic acid, laminarin and curdlan, polyacrylates, polyamides, poly(amide-enamine)s, polyanhydrides, polycaprolactone (PCL), polyglycolic acid (PGA), polyhydroxyalkanoates (PHA), polyhydroxybutyrate (PHB), polylactic acid (PLA), polyurethanes and polyureas, poly(vinyl alcohol) and poly(vinyl acetate) (PVA), pullulan, soy-based plastics, starch and xanthan (Briassoulis, 2004; Flieger et al., 2003).

Polylactic acid (PLA) is a kind of biodegradable plastic which has a wide range of applications, especially food packaging such as cup, bag, bottle, film and wrap. PLA was synthesised by Théophile-Jules Pelouze by the condensation of lactic acid as early as 1845 (Auras et al., 2010), and was developed as biodegradable plastics. PLA is a linear aliphatic polyester consisting of polymerised lactic acid monomers linked by ester bonds. It is synthesised from renewable resources such as corn, cassava, sugar cane, rice and potato through lactic acid fermentation. Biocompatible, compostable, easy-to-fabricate, high mechanical strength and not toxic or carcinogenic substance are favourable properties of PLA.

The use of PLA has received much attention from the viewpoint of environmental protection and solid-waste management. PLA biodegradation is one of the important developments related to those interests. The mechanisms of PLA degradation include photodegradation (Janorkar et al., 2007), hydrolytic degradation (Elsawy et al., 2017) and microbial degradation (Hanphakphoom et al., 2014).

Many studies in microbial degradation since 1997 have isolated pure PLA (pellet, film and granules) degrading actinomycetes. Pranamuda et al. (1997) found microbial degradation of PLA by *Amycolatopsis* sp. HT-32. Since then, PLA degrading actinomycete was found by several researchers such as *Amycolatopsis* sp. KT-s-9 (Tokiwa et al., 1999), *Amycolatopsis* sp. 3118 (Ikura & Kudo, 1999), *Amycolatopsis* sp. K104-1 (Nakamura et al., 2001), *Amycolatopsis* sp. 41 (Pranamuda et al., 2001), *Amycolatopsis*, *Lentzea*, *Kibdelosporangium*, *Streptoalloteichus*, *Saccharothrix* (Jarerat et al., 2002), *Saccharothrix waywayandensis* (Jarerat & Tokiwa, 2003), *Kibdelosporangium aridum* (Jarerat et al., 2003), *Amycolatopsis orientalis* (Jarerat et al., 2006), *Actinomadura* sp. T16-1 (Sukkhum et al., 2009), *Amycolatopsis thailandensis* sp. nov. (Chomchoei et al., 2011), *Pseudonocardia alni* AS4.1531(T) (Konkit et al., 2012) and *Laceyella sacchari* LP175 (Hanphakphoom et al., 2014). However, most studies in the microbial degradation of PLA have focused on pure PLA. A study found that polylactide foil was degraded by *Aspergillus ustus* and *Penicillium verrucosum* (Szumigaj et al., 2008). It shows that the study of PLA-packaging degradation is insufficient.

The Response Surface Methodology (RSM) with Box-Behnken Design is a collection of mathematical and statistical techniques for optimisation of various culture conditions both physical factors (such as agitation rate, pH values and temperature) and culture medium (such as carbon sources, nitrogen sources, mineral elements and growth factors). This method has been successfully applied to enhance the degradation of PLA (Chaisu et al., 2012).

This research, therefore, aimed to isolate, identify and optimise culture conditions of PLA-packaging-degrading actinomycete for bioplastic waste management purposes and the development of the bioplastic industry applications in the future.

MATERIALS AND METHODS

Isolation and Screening of the PLA-packaging-degrading Actinomycete

PLA-packaging was obtained from Dairy Home Co., Ltd, in Nakhon Ratchasima province, Thailand. Prior to use, PLA-packaging was cut (about 1×1 cm) and surface sterilised with 70% (v/v) ethanol and allowed to air dry.

Microbial source samples were collected from liquid biofertilisers, botanical garden soils, cattle pen soils, composts and rubbish dump soils to isolate the PLA-packaging-degrading actinomycete. The samples were collected in pre-sterilised sample bottle following aseptic conditions. The labeled samples were stored at 4°C for further analysis.

The Basal Medium (BM) had the following composition: (per litre of distilled water) 2 g K_2HPO_4; 2 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; and 4 g $(NH_4)_2SO_4$, per 1 L of distilled water (pH value 7.0) (Tomita et al., 1999). The media was autoclaved at 121°C and 15 lbs. pressure. The BM was supplemented with PLA-packaging (1.0 g/L) as sole carbon source.

The sample (10 g) was suspended in 100 mL of BM for 10 min and then settled for 30 min. The supernatant (10 mL) was transferred into 100 mL of BM with 0.1 g of PLA-packaging followed by incubation at 37°C, 180 rpm for 7 days in a rotary shaker. The subcultures were made five times by inoculating 10 mL of the original culture into fresh BM containing PLA-packaging as a carbon source. After five sub-culturing stages, each PLA-packaging was placed on to BM Agar plates for 7 days of incubation at 37°C (Jeon & Kim, 2013). After incubation, colonies that formed around them were purified by re-streaking on Nutrient Agar (NA) contained (g/L): 3 g beef extract; 5 g peptone and 15 g agar per l L of distilled water. Pure isolates were maintained on NA slants at 4C, until used.

PLA-packaging-degrading actinomycete was screened by evaluating the growth in BM with PLA-packaging as a carbon source using the optical density (OD) at wavelength 600 nm (Lee et al., 2013). Spore suspension of the isolates was prepared by streaking each isolate on NA plate and then cultivated for 10 days at 37°C. Spores were harvested by adding sterilised BM to each plate and scrapping the spores using sterilised blade. Spore concentration was adjusted to 1 x 10^7 spores/mL using haemacytometer. Spore suspension (1 mL) of each isolate was inoculated into 100 mL of BM with 0.1 g of PLA-packaging and then incubated at 37°C for 4 weeks under shaking at 180 rpm. BM, BM with PLA-packaging and BM with the cell were used as controls. All treatments were done in triplicate.

Degradation of PLA-packaging by the Isolated Strain

The degradation of PLA-packaging by PLA-packaging-degrading actinomycete was analysed by measuring weight loss and surface changes of the individual samples after 4 weeks of incubation in BM with PLA-packaging as sole carbon source.

PLA-packaging of each treatment was recovered, washed with distilled water 3 times to remove loosely adhered cell and dried in an electronic desiccator (Boekel Dricycler 1344412, Boekel Scientific, Feasterville, Pennsylvania, USA) to ensure a constant weight. Weight loss of the PLA-packaging was calculated according to Equation [1] (Vey et al., 2007).

$$\text{\% Weight loss} = \frac{m_{ini} - m_{dry}}{m_{ini}} \times 100\%$$

Therein m_{ini} is the initial weight of the PLA-packaging before degrading and m_{dry} is the dry weight of the PLA-packaging after degrading. The surface changes of PLA-packaging was examined using a Desktop Scanning Electron Microscopes (MiniSEM) (SEC, model SNE-4500M). Prior to the analysis, the sample was dried overnight and coated with gold to protect the sample morphology against electron beam.

PLA Degradation of the PLA-packaging-degrading Actinomycete

PLA-packaging-degrading actinomycete was studied for the ability to degrade PLA by the clear zone formation around the colony on emulsified PLA agar plate. Emulsified PLA agar was prepared as follows: 0.5 g of PLA pellet (2003D grade, average molecular weight 200,000 g/mol, melting temperature 210°C, purchased from NatureWorks LLC, U.S.A.) was dissolved in 25 mL of dichloromethane and 25 μL of tween 20, and sonicated using an ultrasonicator (VC 505-VC 750; Sonics &

Materials Inc., Newtown, USA) with 50 % power for 5 min in 200 mL of sterilised distilled water. Emulsified PLA solution was mixed with melted BM (added 1 g of yeast extract per 1 L of BM) before pour plate. PLA degradability was investigated after 2 weeks of incubation at 37°C (modified from Penkhrue et al., 2015).

Identification of PLA-packaging-degrading Strain

Macroscopic colony morphology of form, colour, surface, edge, opacity and elevation was observed. The shape, endospore-forming and gram staining characteristic were observed by microscope after gram staining (Wang et al., 2013). The pH values (1 to 10), temperatures (20, 25, 30, 35, 40, 45, 50, 55 and 60°C) and salt concentrations (0 to 14 % NaCl (w/v)) were determined for the range of PLA-packaging-degrading actinomycete growth (Chen et al., 2015).

Genomic DNA of PLA degrading strains was isolated using "Genomic mini kit" (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instruction. The 16S rDNA gene was amplified by PCR using the universal two primers, 20F and 1500R (Brosius et al., 1981) with DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories). The PCR products were analysed by 0.8% (w/v) agarose gel electrophoresis and purified with a GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). Direct sequencing of purified PCR products was carried out on an ABI Prism® 3730 XL DNA Sequence (Applied Biosystem, Foster City, California, USA). Single and double strand 16S rDNA sequencing used 2 (27F or 800R and 518F or 1492R) and 4 (27F, 518F, 800R and 1492R) primers, respectively. The sequences were aligned with EzTaxon-e server (http://eztaxon-e-ezbiocloud.net/; Kim & Park, 2010) together with those of genera possessing sequences similar to those of the isolated strain using CLUSTAL X (version 1.8) (Thompson et al., 1997) in BioEdit program (Hall, 1999). The phylogenetic tree was inferred with the neighbor-joining method of Saitou and Nei (1987) using MEGA version 6.0 (Tamura et al., 2013).

Optimisation of Biomass Production for PLA-packaging Degradation using Response Surface Methodology

Enhancement of the degradation of PLA-packaging by optimisation of *Streptomyces* sp. KKU215 growth conditions was determined. The optimal conditions of the four factors including concentration of yeast extract, initial pH value, temperature and agitation speed for PLA-packaging degradation were investigated by response surface methodology (RSM) with Box-Behnken design using Design-Expert® Software Version 10 (trial version). The ranges of each independent variable are as follows: concentration of yeast extract (1.3-5.3 %), initial pH value (6.0-10.0), temperature (25-45°C) and agitation speed (100-200 rpm), and are shown in Table 1. The cell dry weight was measured after 2 days of cultivation.

Table 1
Experimental code and levels of factors in Box-Behnken design

Independent variables	Code	Levels		
		-1	0	1
Concentration of yeast extract (%)	X_1	1.3	3.3	5.3
Initial pH value	X_2	6.0	8.0	10.0
Temperature (°C)	X_3	25	35	45
Agitation rate (rpm)	X_4	100	150	200

The optimisation results were confirmed by cell dry weight after 2 days of cultivation and degradation of PLA-packaging after 4 weeks of cultivation under the optimal conditions based on the results of RSM.

RESULTS AND DISCUSSION

Isolation and Screening of the PLA-packaging-degrading Actinomycete

Isolation of PLA-packaging-degrading actinomycete among liquid biofertiliser and soil from botanical garden, cattle pen, compost and rubbish dump samples found that only one actinomycete strain from botanical garden soil no. KKU215 exhibited increasing OD in BM supplemented with PLA-packaging as sole carbon source. The increasing OD of KKU215 in the culture medium after 4 weeks as opposed to no increase in OD of the controls (BM, BM with PLA-packaging and BM with KKU215) (Figure 1) demonstrated that KKU215 was able to use PLA as their carbon and energy source.

The growth of tested actinomycete in BM with PLA-packaging as sole carbon source by using the OD at wavelength 600 nm was assessed for their ability to degrade PLA-packaging. The increasing OD of the PBS-2 strain in poly(butylene succinate)-

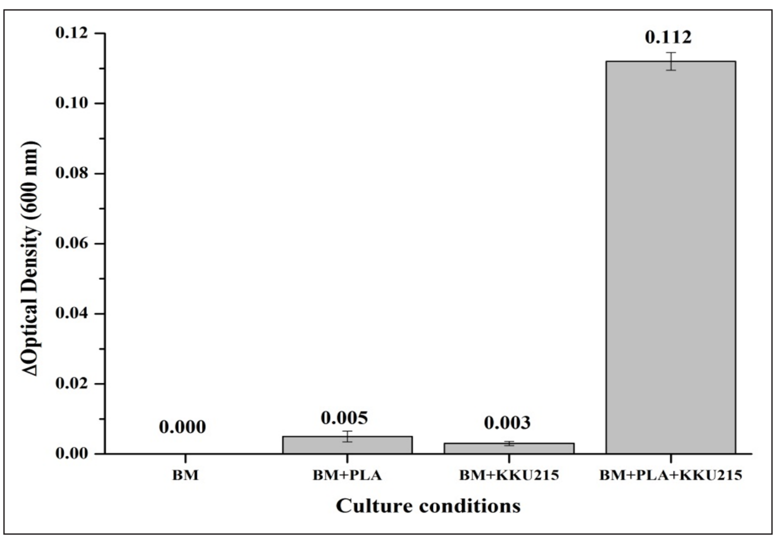


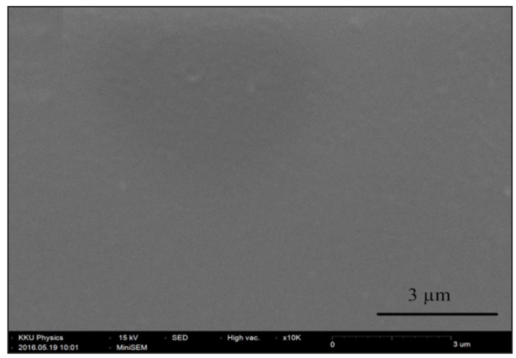
Figure 1. ΔOptical density of PLA-packaging-degrading strain KKU215 in various conditions for 4 weeks at 37°C

tryptone basal liquid medium indicated that PBS-2 could degrade poly(butylene succinate) as carbon source (Lee et al., 2013).

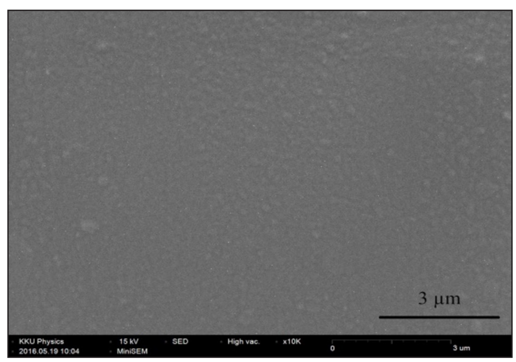
Degradation of PLA-packaging by the Isolated Strain

The percentage of reduction in weight of PLA-packaging by KKU215 was 2.65%, while the weight of the PLA-packaging did not change during incubation in BM without inoculation for 4 weeks. These implied that KKU215 had ability to degrade the PLA-packaging. Although, *Pseudonocardia alni* AS4.1531(T) could degrade PLA film with weight loss of 71.5% within 8 days (Konkit et al., 2012) while weight loss of PLA-packaging in BM by KKU215 was rather lower. This probably was caused by the differences of culture medium which had impact on the growth and all ability. In case of *Pseudonocardia alni* AS4.1531(T), this strain was grown in BM supplemented with 1.0 g yeast extract and 1.0 g gelatin per litre while the KKU215 was grown without any supplementation. Yeast extract had an influence on the growth of *Streptomyces* and their products (Zhou et al., 2017) because yeast extract increases *Streptomyces* mycelium biosynthesis (Suutari et al., 2002). In addition, gelatin could induce PLA degrading activity of actinomycetes (Jarerat et al., 2003; Jarerat & Tokiwa, 2003; Konkit et al., 2012).

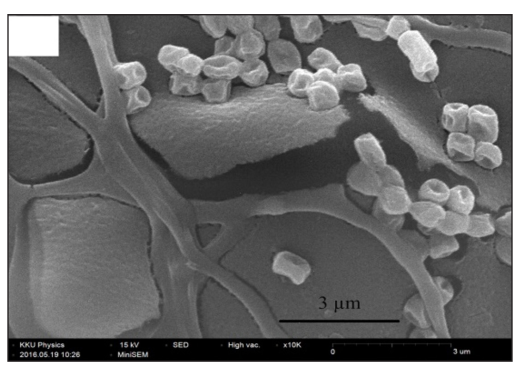
To elucidate the degradation of PLA-packaging induced by KKU215 more clearly, it was important to consider the change of morphology during the degradation. The SEM images of the degraded PLA-packaging were shown in Figures 2a-c. For the inoculated samples, Figure 2c showed a porous structure on the surface and the filaments of KKU215



(a)



(b)



(c)

Figure 2. Scanning electron micrographs of original PLA-packaging surface: (a) after incubation without inoculation of the strain, (b) and after degradation by KKU215, (c) after 4 weeks

able to penetrate the surface of the PLA-packaging. In contrast, the PLA-packaging degraded in control medium (without inoculation of the strain) remained intact (Figure 2b), and very similar to the original PLA-packaging surface (Figure 2a). The penetration of KKU215 filament into PLA-packaging showed the capability to degrade PLA-packaging. Similar to Szumigaj et al. (2008) who investigated the filamentous fungi degradation of commercial poly(lactic acid) foil provided by the Chair of the Food Packaging and Biopolymers Department of the University of Agriculture in Szczecin, Poland. They found that *Aspergillus ustus* and *Penicillium verrucosum* could degrade the PLA food packaging, in which structural changes were observed under the SEM microscope.

The penetration of KKU215 into PLA-packaging and weight loss of PLA-packaging by KKU215 indicated that KKU215 was a PLA-packaging-degrading actinomycete.

PLA Degradation of the PLA-packaging-degrading Actinomycete

The pure PLA degrading ability of KKU215, which is PLA-packaging-degrading actinomycete, was confirmed by the standard clear zone method. KKU215 showed the ability to form clear zone around its colony on emulsified PLA agar plate after 2 weeks of incubation at 37°C (Figure 3). The standard clear zone method was generally used for isolation of biopolymer degrading microorganism including PLA (Hanphakphoom et al., 2014; Penkhrue et al., 2015). The microorganism that could degrade suspended biopolymer in emulsified agar medium would produce clear zone around the colony.

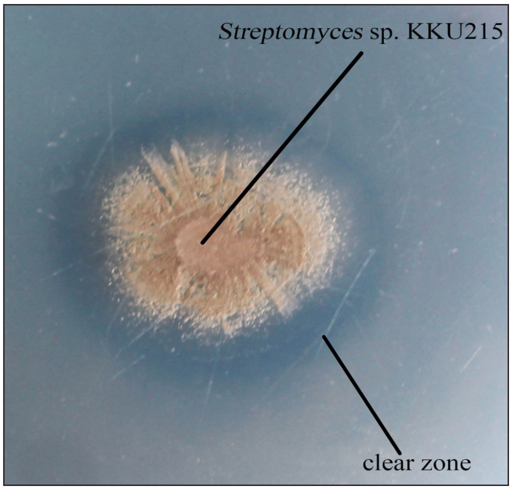


Figure 3. Clear zone formation around the colony of KKU215 on emulsified PLA agar plate for 2 weeks

Identification of PLA-packaging-degrading Strain

KKU215 is a gram-positive, aerobic, filamentous, light white substrate mycelium, grey aerial mycelium, smooth long chain spore. The colony of KKU215 is circular, spore formed in the aerial mycelium and water droplets on colonies. Growth occurs in NA medium at NaCl concentrations, pH values and temperatures at 1 to 6 % (w/v), 6 to 10 and 25 to 45°C, respectively. The phylogenetic relationships of KKU215 were studied based on 16S rDNA gene sequences. The Gene-Bank database was used to search for 16S rDNA sequences similarity to the 16S rDNA sequences of isolates. The results showed that similarity sequencing of KKU215 related to the genus *Streptomyces* and we named it *Streptomyces* sp. KKU215 (Figure 4). The 16S rDNA sequence of *Streptomyces* sp. KKU215 showed similarity with *Streptomyces tendae* (99.31%), *Streptomyces violaceorubidus* (99.31%), *Streptomyces tritolerans* (99.24%), *Streptomyces malachitofuscus* (99.24%), *Streptomyces griseoincarnatus* (99.10%), *Streptomyces variabilis* (99.10%), *Streptomyces erythrogriseus* (99.10%), *Streptomyces rubrogriseus* (99.10%), *Streptomyces griseorubens* (99.10%), *Streptomyces flavoviridis* (99.10%), *Streptomyces labedae* (99.09%), *Streptomyces pilosus* (99.09%) and *Streptomyces lienomycini* (99.02%). KKU215 formed a cluster within the genus *Streptomyces* and similarity more than 99% with numerous *Streptomyces*. Nevertheless, these *Streptomyces* species had not been reported on PLA degradation yet. Therefore,

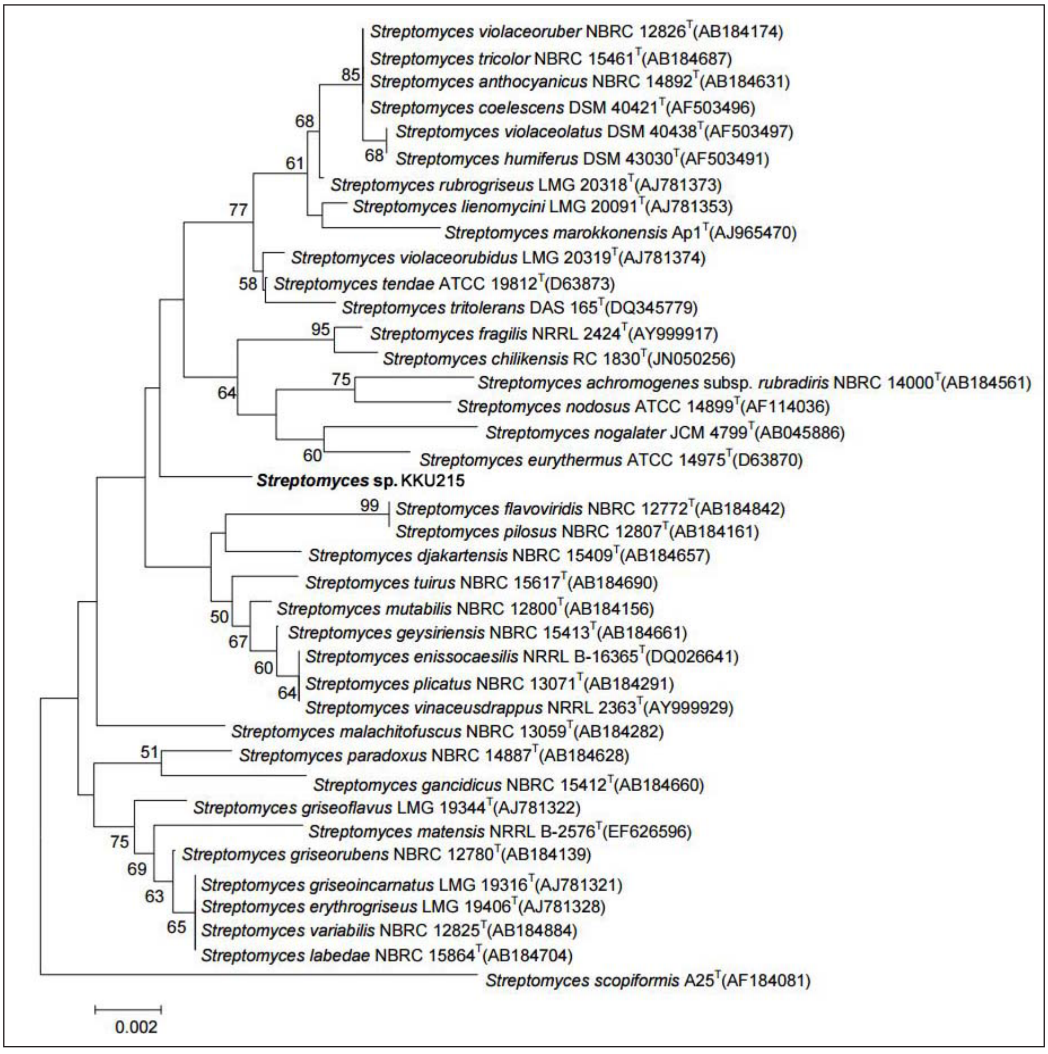


Figure 4. Phylogenetic tree based on 16S rDNA gene sequences of KKU215 and related species of the genus *Streptomyces*. Numbers at nodes are percentage bootstrap values based on 1000 replicates

Streptomyces sp. KKU215 is a novel strain for PLA degradation.

Optimisation of Biomass Production for PLA-packaging Degradation using Response Surface Methodology

A Box-Behnken design under RSM was used to analyse the main and interactive effect of concentration of yeast extract, initial pH value, temperature and agitation speed to reach the optimum level in relation to dry weight. Twenty-eight experiments and its responses are shown in Supplementary Table 1.

The experimental results gained from Box-Behnken design were fitted to a second order polynomial model to explain the dependence of dry weight on the four factors. The regression equation is shown as follow:

$$Y = -2892.57853 + (84.41313X_1) + (338.44792X_2) + (66.18875X_3) + (7.32177X_4) + (0.5625X_1X_2) + (0.1125X_1X_3) + (0.07825X_1X_4) + (0.6125X_2X_3) - (0.03475X_2X_4) - (0.0052X_3X_4) - (16.09479X_1^2) - (23.13229X_2^2) - (1.04217X_3^2) - (0.023917X_4^2) \quad [2]$$

where Y is the predicted dry weight, and X_1, X_2, X_3 and X_4 are the coded variables of concentration of yeast extract, initial pH value, temperature and agitation speed, respectively.

The adequacy of the model was checked through analysis of variance (ANOVA). The results of the second order response surface model fitting in the form of ANOVA are given in Supplementary Table 2. The model was found highly significant having Fischer test value of 25.67 and *P*-value of <0.0001. The coefficient of determination R^2 showed the appropriateness of the adequate model. The coefficient of determination $R^2 = 0.9651$ or 96.51% indicated that about 3.49% variations were not determined by the model. The adjusted determination coefficient $R^2 = 0.9275$ or 92.75% also showed that the model was highly significant. Consequently, the regression model was believed to accurately and reliably predict and analyse the dry weight of *Streptomyces* sp. KKU215.

The linear effect of pH (p-value = 0.0002) and temperature (p-value = 0.0007) was highly significant. The quadratic effect of all factors showed great impact on the dry weight according to the significance of corresponding p-values (<0.0001), indicating that the change of the response value (dry weight) was complex. The effect of the factors on the dry weight of *Streptomyces* sp. KKU215 was not a simple linear relationship but a significant surface effect (Yun et al., 2018). The interaction effect of all four factors was not significant, indicating that there are no interactions between variables.

Regression models generated the three-dimensional response surface plots to determine the impact of independent variables and interaction effect of each variable , and discover the optimal level of each variable for maximal response (Figures 5 and 6). Each of 3D response surface graphs and contour plots was generated using two test variables at one time while keeping the other two variables at their centre value. The convex response surfaces suggest that there are well-defined optimal variables. Every response surface plot clearly indicated that the optimal condition should occur with medium levels of concentration of yeast extract, initial pH value, temperature and agitation speed.

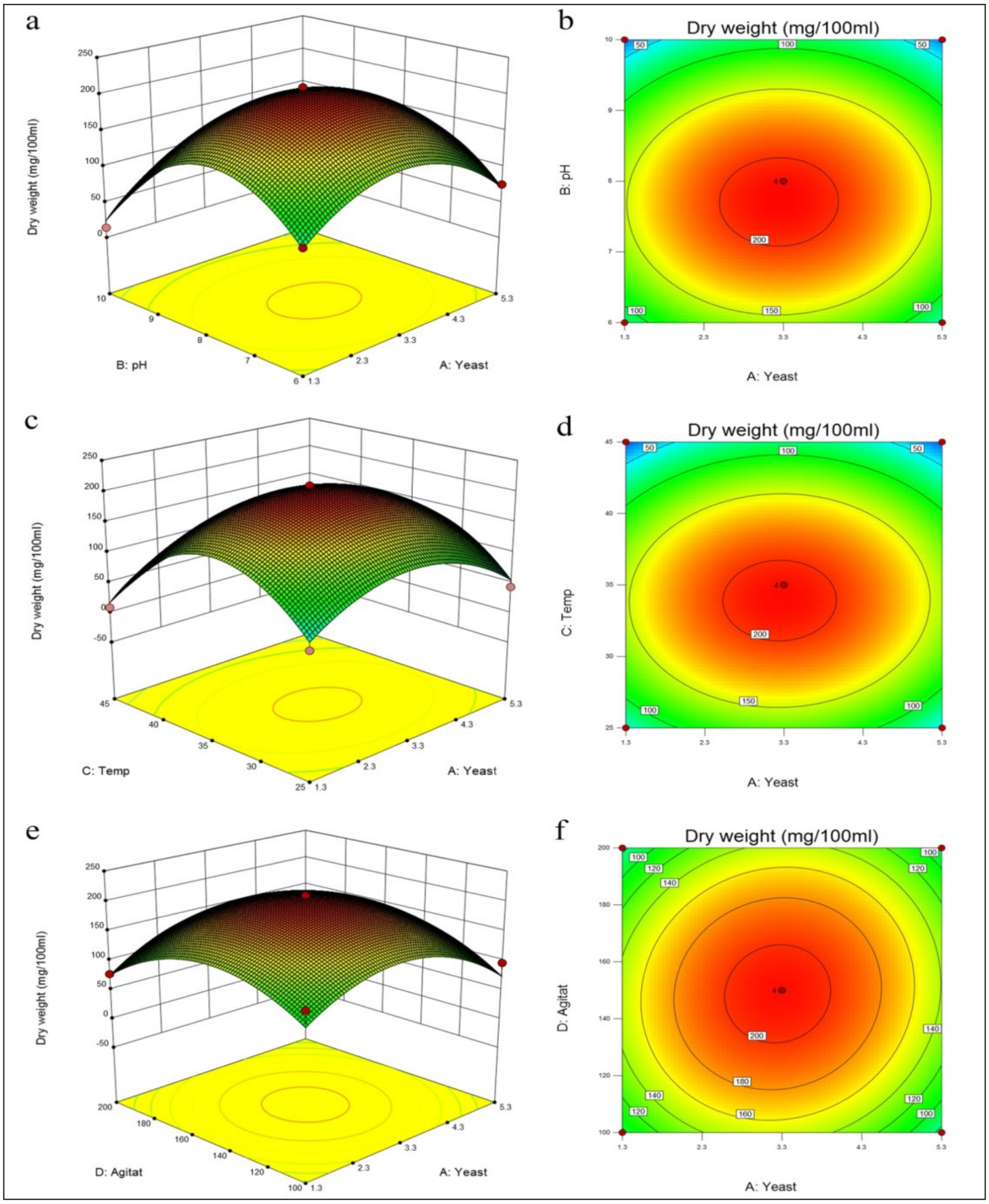


Figure 5. Response surface plots (3D; a, c, e) and contour plots (2D; b, d, f) showing the individual and interactive effects of variables on the dry weight of *Streptomyces* sp. KKU215 [(a, b) effects of concentration of yeast extract and pH; (c, d) effects of concentration of yeast extract and temperature; (e, f) effects of concentration of yeast extract and agitation speed]

By referring to Figure 5, the increase of yeast extract concentration from 1.30% to 3.26% (optimal concentration of yeast extract) increased the dry weight of *Streptomyces* sp. KKU215. Yeast extract is an excellent organic nitrogen source, contains high protein and amino acid content, vitamins, minerals and growth factors that are essential for *Streptomyces* growth (Chen et al., 2012). However, an increase in concentration of yeast extract higher than optimal condition decreased the dry weight.

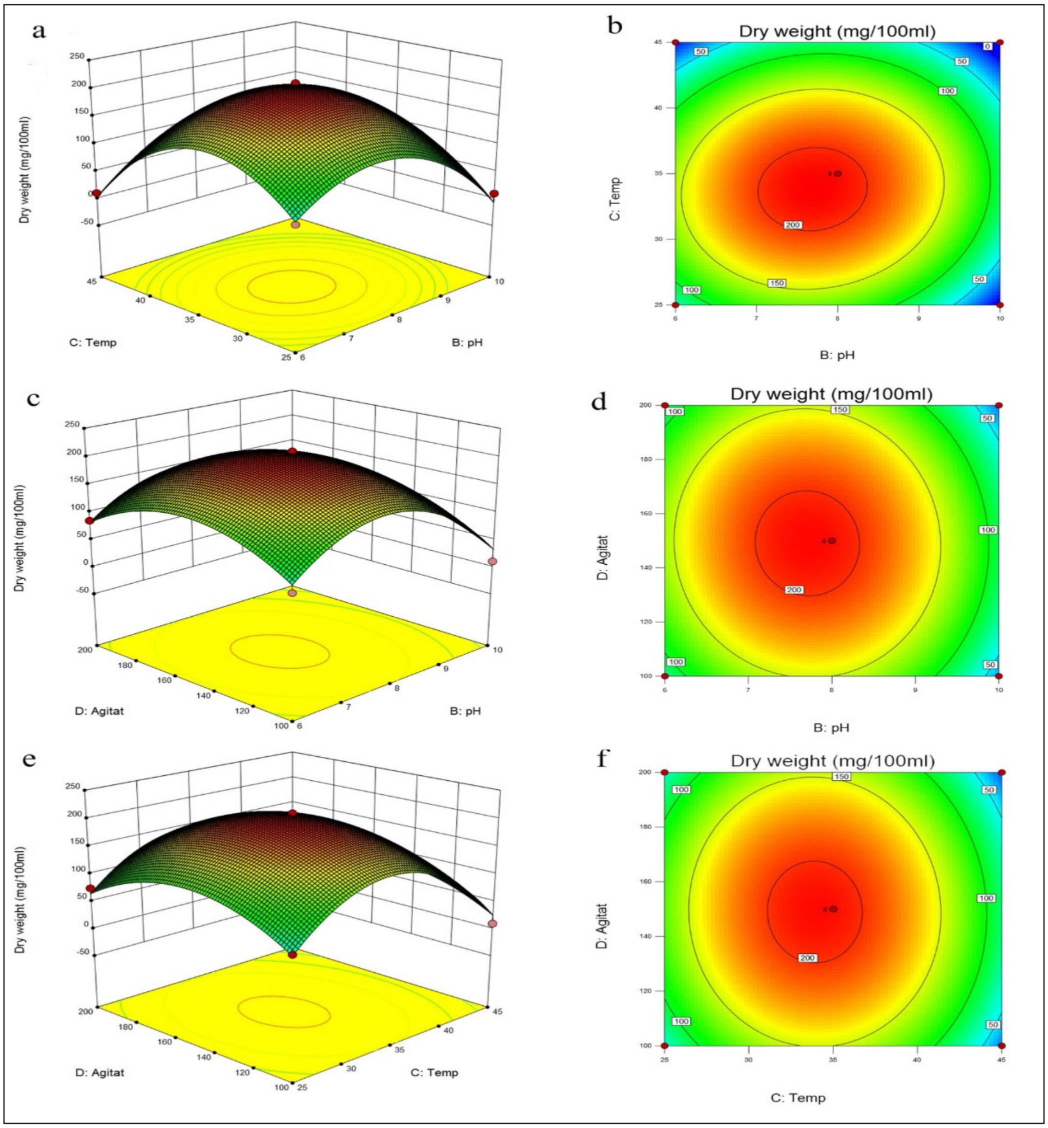


Figure 6. Response surface plots (3D; a, c, e) and contour plots (2D; b, d, f) showing the individual and interactive effects of variables on the dry weight of *Streptomyces* sp. KKU215 [(a, b) effects of pH and temperature; (c, d) effects of pH and agitation speed; (e, f) effects of temperature and agitation speed]

On the one hand, *Streptomyces* sp. KKU215 could grow best at neutral initial pH value (7.69). On the other hand, the acidic and alkaline initial pH values could decrease the dry weight of *Streptomyces* sp. KKU215 (Figures 5a, 5b, 6a, 6b, 6c and 6d). The optimal pH of *Streptomyces* was neutral (pH 6.5-8.0) (Liu et al., 2018; Praveen Kumar et al., 2017; Srivastava et al., 2018; Yamamura et al., 2014).

Dry weight of *Streptomyces* sp. KKU215 increased with increasing temperature up to 33.66°C (optimal temperature). Above the optimal temperature, a negative effect of the temperature on *Streptomyces* sp. KKU215 dry weight was discovered (Figures 5c, 5d, 6a, 6b, 6e and 6f). The temperatures were higher or lower than optimal temperature reduced growth, metabolite production and enzyme activity of *Streptomyces* (Praveen Kumar et al., 2017; Srivastava et al., 2018; Yun et al., 2018).

Agitation speed affects oxygen transfer rate, which plays an important role in aerobic microbial metabolism. Nevertheless, different microorganisms have different oxygen requirements. In this study, the optimal agitation speed of *Streptomyces* sp. KKU215 was 149.13 rpm (Figures 5e, 5f, 6c, 6d, 6e and 6f) as reported by Viana et al. (2010) who found that the highest clavulanic acid production by *Streptomyces* DAUFPE 3060 was achieved at agitation speed of 150 rpm.

Overall, the model predicted that the optimal values of the test factors including concentration of yeast extract, initial pH value, temperature and agitation speed were 3.26%, 7.69, 33.66°C and 149.13 rpm, respectively. The predicted maximum dry weight of *Streptomyces* sp. KKU215 was 210.49 mg/100 mL (Table 2).

Table 2
Optimal values of the test variables, and predicted maximum dry weight

Variables	Value
X_1: Concentration of yeast extract (%)	3.26
X_2: Initial pH value	7.69
X_3: Temperature (°C)	33.66
X_4: Agitation rate (rpm)	149.13
predicted maximum dry weight (mg/100 mL)	210.49

In order to validate the model, the optimisation results were confirmed by cell dry weight after 2 days of cultivation and degradation of PLA-packaging after 4 weeks of cultivation under the optimal conditions based on the results of RSM. The model predicted a maximum response of 210.49 mg/100 mL of *Streptomyces* sp. KKU215 dry weight. At optimal conditions, a maximum *Streptomyces* sp. KKU215 dry weight of 209.25 mg/100 mL was obtained. Thus, the model proved adequate. Moreover, PLA-packaging weight loss under the optimal conditions by *Streptomyces* sp. KKU215 was 84.04%.

CONCLUSION

KKU215, which was isolated from botanical garden soil, was able to degrade PLA-packaging including PLA. Phylogenetic study by 16SrRNA sequence analysis of KKU215 indicated that it related to the genus *Streptomyces*. *Streptomyces* sp.

KKU215 was a novel PLA-packaging-degrading actinomycete. The optimal conditions of concentration of yeast extract, initial pH value, temperature and agitation speed were determined through RSM to be 3.26%, 7.69, 33.66°C and 149.13 rpm, respectively. The optimisation resulted in PLA-packaging weight loss of 84.04%, which was 81.39% higher than that before optimisation (2.65%). The weight loss of PLA-packaging in optimal conditions decreased almost thirty-two-fold than before optimisation. Based on the present findings, *Streptomyces* sp. KKU215 can be used as a potent microbial strain to degrade PLA-packaging. The environmental degradation of PLA-packaging by *Streptomyces* sp. KKU215 will be investigated in further study.

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REFERENCES

Auras, R., Lim, L. T., Selke, S. E. M., & Tsuji, H. (2010). Preface. In R. Auras, L. T. Lim, S. E. M. Selke, & H. Tsuji (Eds.), *Poly(lactic acid): Synthesis, structures, properties, processing, and applications* (pp. xvii-xix). Hoboken, USA: John Wiley & Sons, Inc.

Briassoulis, D. (2004). An overview on the mechanical behaviour of biodegradable agricultural films. *Journal of Polymers and the Environment*, *12*(2), 65–81.

Brosius, J., Dull, T. J., Sleeter, D. D., & Noller, H. F. (1981). Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *Journal of Molecular Biology*, *148*(2), 107-127.

Chaisu, K., Charles, A. L., Guu, Y. K., & Chiu, C. H. (2012). Optimization of polylactic acid (PLA) plastic degradation by *Aneurinibacillus migulanus* using response surface methodology. In *International Conference on Biological and Life Sciences* (Vol. 40, pp. 22-27). Singapore: International Association of Computer Science and Information Technology (IACSIT) Press.

Chen, X., Wang, Z., Wang, Y. Z., Jiang, F. T., Li, D. S., & Wang, J. H. (2012). Effects of yeast extract on poly-ε-lysine production in batch culture of *Streptomyces albulus*. In *Advanced materials research* (Vol. 343, pp. 1023-1028). Zürich, Switzerland: Trans Tech Publications.

Chen, Y. G., Gu, F. L., Xu, F., He, S. Z., & Fang, Y. M. (2015). *Bacillus vanillea* sp. nov., isolated from the cured vanilla bean. *Current Microbiology*, *70*(2), 235-239.

Chomchoei, A., Pathom-aree, W., Yokota, A., Khanongnuch, C., & Lumyong, S. (2011). *Amycolatopsis thailandensis* sp. nov., a poly(L-lactic acid)-degrading actinomycete, isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*, *61*(4), 839-843.

Elsawy, M. A., Kim, K., Park, J., & Deep, A. (2017). Hydrolytic degradation of polylactic acid (PLA) and its composites. *Renewable and Sustainable Energy Reviews*, *79*, 1346–1352.

Flieger, M., Kantorová, M., Prell, A., Rezanka, T., & Votruba, J. (2003). Biodegradable plastics from renewable sources. *Folia Microbiologica*, *48*(1), 27–44.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series* (Vol. 41, No. 41, pp. 95-98). London, England: Information Retrieval Ltd.

Hanphakphoom, S., Maneewong, N., Sukkhum, S., Tokuyama, S., & Kitpreechavanich, V. (2014). Characterization of poly(L-lactide)-degrading enzyme produced by thermophilic filamentous bacteria *Laceyella sacchari* LP175. *Journal of General and Applied Microbiology*, *60*(1), 13-22.

Ikura, Y., & Kudo, T. (1999). Isolation of a microorganism capable of degrading poly-(L-lactide). *The Journal of General and Applied Microbiology*, *45*(5), 247–251.

Janorkar, A. V., Metters, A. T., & Hirt, D. E. (2007). Degradation of poly(L-lactide) films under ultraviolet-induced photografting and sterilization conditions. *Journal of Applied Polymer Science*, *106*(2), 1042–1047.

Jarerat, A., & Tokiwa, Y. (2003). Poly(L-lactide) degradation by *Saccharothrix waywayandensis*. *Biotechnology Letters*, *25*(5), 401–404.

Jarerat, A., Pranamuda, H., & Tokiwa, Y. (2002). Poly(L-lactide) degrading activity in various actinomycetes. *Macromolecular Bioscience*, *2*(9), 420–428.

Jarerat, A., Tokiwa, Y., & Tanaka, H. (2003). Poly(L-lactide) degradation by *Kibdelosporangium aridum*. *Biotechnology Letters*, *25*(23), 2035–2038.

Jarerat, A., Tokiwa, Y., & Tanaka, H. (2006). Production of poly(L-lactide)-degrading enzyme by *Amycolatopsis orientalis* for biological recycling of poly(L-lactide). *Applied Microbiology and Biotechnology*, *72*(4), 726-731.

Jeon, J. H., & Kim, N. M. (2013). Biodegradation of poly(L-lactide) (PLA) exposed to UV irradiation by a mesophilic bacterium. *International Biodeterioration and Biodegradation*, *85*, 289-293.

Kim, M. N., & Park, S. T. (2010). Degradation of poly(L-lactide) by a mesophilic bacterium. *Journal of Applied Polymer Science*, *117*(1), 67–74.

Konkit, M., Jarerat, A., Khanongnuch, C., Lumyong, S., & Pathom-aree, W. (2012). Poly(lactide) degradation by *Pseudonocardia alni* AS4.1531(T). *Chiang Mai Journal of Science*, *39*(1), 128-132.

Lee, J. S., Park, H. E., Han, H. Y., Kim, O. Y., & Park, W. S. (2013). Isolation of a marine bacterium capable of biodegrading poly(butylene succinate). *Journal of Fisheries and AquaticSciences*, *16*(1), 41-44.

Liu, X., Sun, X., Wang, T., Zhang, X., Tian, X., Zhuang, Y., & Chu, J. (2018). Enhancing candicidin biosynthesis by medium optimization and pH stepwise control strategy with process metabolomics analysis of *Streptomyces* ZYJ-6. *Bioprocess and Biosystems Engineering*, *41*(12), 1743–1755.

Nakamura, K., Tomita, T., Abe, N., & Kamio, Y. (2001). Purification and Characterization of an extracellular poly(L-lactic acid) depolymerase from a soil isolate, *Amycolatopsis* sp. strain K104-1. *Applied and Environmental Microbiology*, *67*(1), 345–353.

Penkhrue, W., Khanongnuch, C., Masaki, K., Pathom-aree, W., Punyodom, W., & Lumyong, S. (2015). Isolation and screening of biopolymer-degrading microorganisms from northern Thailand. *World Journal of Microbiology and Biotechnology*, *31*(9), 1431-1442.

Pranamuda, H., Tokiwa, Y., & Tanaka, H. (1997). Polylactide degradation by an *Amycolatopsis* sp. *Applied and Environmental Microbiology*, *63*(4), 1637–1640.

Pranamuda, H., Tsuchii, A., & Tokiwa, Y. (2001). Poly(L□lactide) degrading enzyme produced by *Amycolatopsis* sp. *Macromolecular Bioscience*, *1*(1), 25–29.

Praveen Kumar, P., Sagaya Jansi, R., Saravana Kumar, P., Nimal Christhudas, I. V. S., Preetam Raj, J. P., Vijayakumar, A., … Ignacimuthu, S. (2017). Optimization of biosynthesis parameters, partial purification and characterization of extracellular lipase from soil derived *Streptomyces* sp. Loyola Lipase-1. *Biocatalysis and Agricultural Biotechnology*, *12*, 241–247.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406-425.

Srivastava, A., Singh, V., Haque, S., Pandey, S., Mishra, M., Jawed, A., … Tripathi, C. K. M. (2018). Response surface methodology-genetic algorithm based medium optimization, purification, and characterization of cholesterol oxidase from *Streptomyces rimosus*. *Scientific Reports*, *8*(1), 10913.

Sukkhum, S., Tokuyama, S., Tamura, T., & Kitpreechavanich, V. (2009). A novel poly(L-lactide) degrading actinomycetes isolated from Thai forest soil, phylogenic relationship and the enzyme characterization. *Journal of General and Applied Microbiology*, *55*(6), 459–467.

Suutari, M., Lignell, U., Hyvärinen, A., & Nevalainen, A. (2002). Media for cultivation of indoor streptomycetes. *Journal of Microbiological Methods*, *51*(3), 411–416.

Szumigaj, J., Zakowska, Z., Klimek, L., Rosicka-Kaczmarek, J., & Bartkowiak, A. (2008). Assessment of polylactide foil degradation as a result of filamentous fungi activity. *The Polish Journal of Environmental Studies*, *17*(3), 335-341.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725-2729.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*(24), 4876-4882.

Thompson, R. C., Moore, C. J., Vom Saal, F. S., & Swan, S. H. (2009). Plastics, the environment and human health: Current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2153-2166.

Tokiwa, Y., Konno, M., & Nishida, H. (1999). Isolation of silk degrading microorganisms and its poly(L-lactide) degradability. *Chemistry Letters*, *28*(4), 355-356.

Tomita, K., Kuroki, Y., & Nagai, K. (1999). Isolation of thermophiles degrading poly(L-lactic acid). *Journal of Bioscience and Bioengineering*, *87*(6), 752–755.

Vey, E., Miller, A. F., Claybourn, M., & Saiani, A. (2007). *In vitro* degradation of poly(lactic-*co2*-glycolic) acid random copolymers. *Macromolecular Symposia*, *251*(1), 81-87.

Viana, D. A., Carneiro-Cunha, M. N., Araújo, J. M., Barros-Neto, B., Lima-Filho, J. L., Converti, A., … Porto, A. L. F. (2010). Screening of variables influencing the clavulanic acid production by *Streptomyces* DAUFPE 3060 strain. *Applied Biochemistry and Biotechnology*, *160*(6), 1797-1807.

Wang, Y., Yuan, Y., & Yu, Z. (2013). *Bacillus borbori* sp. nov., isolated from an electrochemically active biofilm. *Current Microbiology*, *67*(6), 718-724.

Yamamura, H., Ashizawa, H., Hamada, M., Hosoyama, A., Komaki, H., Otoguro, M., … Hayakawa, M. (2014). *Streptomyces hokutonensis* sp. nov., a novel actinomycete isolated from the strawberry root rhizosphere. *The Journal of Antibiotics*, *67*(6), 465-470.

Yun, T. Y., Feng, R. J., Zhou, D. B., Pan, Y. Y., Chen, Y. F., Wang, F., … Xie, J. H. (2018). Optimization of fermentation conditions through response surface methodology for enhanced antibacterial metabolite production by *Streptomyces* sp. 1-14 from cassava rhizosphere. *PLoS ONE*, *13*(11), 1-21.

Zhou, Y., Sun, Y. B., He, H. W., Feng, J. T., Zhang, X., & Han, L. R. (2017). Optimization of medium compositions to improve a novel glycoprotein production by *Streptomyces kanasenisi* ZX01. *AMB Express*, *7*(6), 1-9.

APPENDIX

Supplementary Table 1
The Box–Behnken experimental design and results

Run No.	Model	Concentration of yeast extract (%)	Initial pH value	Temperature (°C)	Agitation rate (rpm)	Dry weight (mg/100 mL)	
						Actual value	Predicted value
1	+00+	5.3	8	35	200	83.0	84.6
2	0+-0	3.3	10	25	150	10.0	-6.5
3	0000	3.3	8	35	150	204.0	207.0
4	+-00	5.3	6	35	150	76.0	71.7
5	0-0+	3.3	6	35	200	86.0	82.6
6	0-0-	3.3	6	35	100	68.1	81.1
7	00--	3.3	8	25	100	68.1	65.9
8	-+00	1.3	10	35	150	14.0	23.9
9	0++0	3.3	10	45	150	2.0	-27.4
10	+00-	5.3	8	35	100	97.0	74.5
11	-0+0	1.3	8	45	150	9.0	16.7
12	00-+	3.3	8	25	200	75.5	65.5
13	0--0	3.3	6	25	150	68.0	72.4
14	-00-	1.3	8	35	100	123.3	96.7
15	0+0+	3.3	10	35	200	15.0	21.3
16	-0-0	1.3	8	25	150	54.0	66.6
17	0+0-	3.3	10	35	100	11.0	33.7
18	00+-	3.3	8	45	100	10.0	25.6
19	00++	3.3	8	45	200	7.0	14.9
20	0000	3.3	8	35	150	210.0	207.0
21	++00	5.3	10	35	150	15.0	21.9
22	--00	1.3	6	35	150	84.0	82.8
23	+0-0	5.3	8	25	150	44.0	55.6
24	0000	3.3	8	35	150	208.0	207.0
25	0-+0	3.3	6	45	150	11.0	2.4
26	-00+	1.3	8	35	200	78.0	75.5
27	+0+0	5.3	8	45	150	8.0	14.6
28	0000	3.3	8	35	150	206.0	207.0

Supplementary Table 2
Analysis of variance (ANOVA) for the regression equation

Source	SS	df	MS	*F*-value	*P*-value
Model	115700.00	14	8262.47	25.670	<0.0001
Concentration of yeast extract	128.71	1	128.71	0.400	0.5381
pH	8861.77	1	8861.77	27.540	0.0002
Temperature	6192.56	1	6192.56	19.240	0.0007
Agitation rate	90.75	1	90.75	0.280	0.6044
Concentration of yeast extract* pH	20.25	1	20.25	0.063	0.8059
Concentration of yeast extract* Temperature	20.25	1	20.25	0.063	0.8059
Concentration of yeast extract* Agitation rate	244.92	1	244.92	0.760	0.3988
pH* Temperature	600.25	1	600.25	1.870	0.1952
pH* Agitation rate	48.30	1	48.30	0.150	0.7047
Temperature* Agitation rate	27.04	1	27.04	0.084	0.7765
Concentration of yeast extract* Concentration of yeast extract	24868.06	1	24868.06	77.270	<0.0001
pH*pH	51369.88	1	51369.88	159.620	<0.0001
Temperature* Temperature	65166.68	1	65166.68	202.490	<0.0001
Agitation rate* Agitation rate	21450.26	1	21450.26	66.650	<0.0001
Lack of Fit	4163.68	10	416.37	62.460	0.0029
Pure Error	20.00	3	6.67		
Cor Total	119900.00	27			

R^2 = 0.9651
Adjusted R^2 = 0.9275
Predicted R^2 = 0.7996

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Indigenous Mycorrhizal Seed-coating Inoculation on Plant Growth and Yield, and NP-uptake and Availability on Maize-sorghum Cropping Sequence in Lombok's Drylands

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ABSTRACT

By improving the nutrient uptake and transport, an indigenous arbuscular mycorrhizal fungal (AMF) is expected to improve crops' performance in sandy drylands of North Lombok (Indonesia) during dry seasons. A field experiment was designed with Randomized Complete Block Design and four replications to examine the benefits of mycorrhiza at varying doses of plant nutrition (nitrogen and phosphorus). Total of 1 kg of the AMF inoculum was applied to 20 kg maize seeds in different fertilization packages of cattle manure (15, 12, 9 and 6 ton/ha) and inorganic fertilizers (80, 60, 40 and 20% NPK recommended dose). A 100% NPK recommended dose was used as the control (200 kg/ha Phonska and 300 kg/ha Urea). After harvest of maize at 100 days after seeding (DAS) and field cleaning from maize debris, sorghum seeds were then planted in cropping cycle 2 with no additional fertilization and inoculum. Results indicated that the AMF applications to the maize-sorghum cropping sequence increased the AMF colonization rate, soil N and P status and uptake, and dry biomass (root, shoot, and grain). The highest correspondence was observed in the crops which utilized a combination of 60% NPK and 12 ton/ha cattle manure, and the performance was higher at 100 days after seeding. The number of AMF spores increased over the time where colonization rates were found higher in roots of sorghum (60-81%) than maize (55-75%). This study suggests that AMF inoculation increases the plant yield and improves soil nutrient availability

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which is very advantageous for the growth of the maize-sorghum subsequent crop in Lombok's drylands.

Keywords: Arbuscular mycorrhizal fungi (AMF), cattle manure, maize-sorghum cropping sequence, plant nutrition, seed coating

INTRODUCTION

The northern part of North Lombok regency (Indonesia) is dominated by drylands with sandy soils texture. With a very short and low number of rainy days (December to April, 100-200 mm) per wet month or no rain during the long dry seasons (May to November), no food crops can be cultivated normally especially in the areas without deep wells. Moreover, inadequate phosphorus (P) availability is also one of the factors limiting the productivity of maize and other food crops in the dryland of North Lombok. Maize crop requires very high P; for example, for the production of 10 ton/ha, maize crops need 102 kg/ha of P_2O_5 and 76% of it is transported into the seeds (Calderón-Vázquez et al., 2009). In contrast, plant roots can take up P at only about 8-13% of the amount of P fertilizer applied (Supardi, 1996). Thus, to cope with the unavailability of soil water and P and other essential nutrients in drylands of North Lombok, arbuscular mycorrhizal fungal (AMF) symbiosis has been used as it is expected to improve the performance of food crops especially during the dry seasons. Many have reported that the soil fungi, in symbiosis with their host plants, can help plants to improve water retention and make their host plants more tolerant to drought (Augé, 2004). AMF colonization also increases nutrient uptake from soils and enhances growth and yield of the host plants although it depends on the species of AMF colonizing the host plants (Marulanda et al., 2003).

The most common findings show that the external hyphae of AMF improve P uptake and transport the nutrient to root cortex of their host (Azcón et al., 2003; Feng et al., 2003; George et al., 1995; Koide & Kabir, 2000; Tarafdar & Marschner, 1994; Tawaraya et al., 2006). Although there is no growth improvement of the host plants due to the AMF symbiosis, the amount of P uptake through AMF (mycorrhizal pathway) can be higher than through the host roots or the direct pathway (Smith & Read, 2010). AMF can also help their hosts to increase the uptake of other essential nutrients such as N, K, Ca, Mg, S, Cu, Zn, Mn, Fe and B when compared with non-mycorrhizal plants (Dhillion & Ampornpan, 1992; Hawkins et al., 2000; Leigh et al., 2009; Menge, 1983). Most of these capabilities of the AM plants are due to the extensive growth of AMF external hyphae beyond the host roots (Drew et al., 2003; Zhu et al., 2001). The length ratio of AMF hyphae to roots in soil can be 100:1 or higher and with the external hyphae, the mycorrhizal roots can explore further (George et al., 1995). By applying the Plenchette's mycorrhizal dependency formula to the shoot dry weight of maize and sorghum (Plenchette et al., 1983), the results showed ~~a~~ high mycorrhizal dependency, both for maize (up to 70.12%) and for sorghum (up to 79.42%) (Guo et al., 2013;

Tawaraya, 2003). The findings of the study indicate that the higher the dependent rate is on AMF symbiosis, the more the dry matter is produced of the crops. With the high porosity and low water retention capacity on dryland and sandy areas (Bruand et al., 2005) in most areas of North Lombok, the levels of crop dependence on symbiosis with AMF could be higher. Moreover, the symbiosis of crops with AMF could significantly reduce nutrient loss from soil through leaching (Cavagnaro et al., 2015).

The establishment of AM symbiosis can be done through inoculation with AMF propagules. Our previous study (Astiko et al., 2013a) showed that the indigenous AMF inoculation in maize plants in sandy soils had positive implications for the improvement of soil properties by increasing the rates of nutrient uptake by maize crop from the soil and improving its grain yield. Our research group has also shown the benefit of this local inoculation in increasing the growth of soybean and its yield by improving P uptake from the soils, compared to those without AMF inoculation (Astiko et al., 2013b). However, as founded from other studies, the development of AMF in the soil and its contribution to growth, yield and nutrient uptake of host plants could be influenced by the order of plant species cultivated in sequence in the cropping system (Johnson et al., 1992; Vivekanandan & Fixen, 1991). The studies by Johnson et al. (1992) as well as Vivekanandan and Fixen (1991) reported that the P uptake and the AMF colonization were higher on maize crops grown following soybean compared to when maize crops were grown following maize or barley.

From the previous study on maize-soybean cropping patterns, it was found that the AMF inoculated in maize crops grown in the first cycle increased root colonization and AMF sporulation which was very advantageous for the growth of the following crop in the cropping sequence (Astiko, 2013). Different AMF populations (colonization and spore counts) were also found between cropping seasons or between crop species in the same cropping season in Central Lombok, Indonesia (Wangiyana et al., 2006). The present study examined the effects of several combinations of fertilizer packages consisting of indigenous AMF bio-fertilizer and varying doses of organic and NPK fertilizers applied to maize crops on N and P status in soil, and uptake by maize and subsequent sorghum crops as well as the growth and yield components of the maize and sorghum crops in a maize-sorghum cropping sequence on drylands in North Lombok, Indonesia.

MATERIALS AND METHODS

Design of the Experiment

The field experiment of maize-sorghum cropping sequence in this study was arranged according to the Randomized Complete Block Design (RCBD) with four replications (blocks). The study was carried out in the Akar-Akar village located in North Lombok regency, Indonesia, from January to August 2016. Treatments involving the use of five fertilizer packages consisting of different combinations of organic, inorganic and indigenous AMF bio-fertilizer were applied only to the maize crop in the first cropping cycle of the maize-sorghum cropping sequence. The 100% NPK-only recommended dose (D_0) is the farmer's practice of dose for maize by the locals. The NPK's doses were decreased and had been replaced by cattle manure in varying fertilization packages (D_1, D_2, D_3, D_4), and added with AMF as listed in Table 1.

Table 1
The mycorrhizal-based fertilization packages tested and applied to maize only in the maize-sorghum cropping sequence

Fertilization packages	Doses of the packages applied to maize plants only (in the cropping cycle 1)	Sorghum (cropping cycle 2)
D_0	NPK only at 100% the recommended doses (RD), i.e. 200 kg/ha Phonska (NPK 15:15:15) and 300 kg/ha Urea	No fertilizer applied
D_1	NPK at 80% RD + 15 ton/ha cattle manure + AMF	No fertilizer applied
D_2	NPK at 60% RD + 12 ton/ha cattle manure + AMF	No fertilizer applied
D_3	NPK at 40% RD + 9 ton/ha cattle manure + AMF	No fertilizer applied
D_4	NPK at 20% RD + 6 ton/ha cattle manure + AMF	No fertilizer applied

A piece of land used in this study was of a sandy soil type (69% sand, 29% silt and 2% clay, containing an average of 421 AMF spores per 100 g soil), typical of North Lombok area. The land is located at a geographic position of -8.221650, 116.350283, and measured with 13.82 mg/kg of available P, 0.01% Total N, 0.57 cmol/kg of available K, 7.31 cmol/kg of Ca, and 1.21% of C-organic. After being cultivated using minimum tillage and cleared of weeds, the land was splitted into 20 treatment plots of 7m x 5m based on the RCBD plotting layout. The different fertilization packages consisted of AMF inoculum, organic fertilizer (cattle manure), and inorganic fertilizer (NPK and Urea), were applied only to the maize crop in the first cropping cycle.

An indigenous AMF inoculum, *Glomus mosseae* (the M_{AA01} mycorrhizal isolate including the hyphae and the mycorrhizal spores), which was originally isolated from dryland area (1,500 spores per 20 g of soils) in Akar-Akar village of North Lombok, was applied through seed-coating of maize seeds prior to seeding. Before application, 1 kg of the AMF inoculum was mixed with 300 g of charcoal powder and 90 g of tapioca flour as the carrier materials. Then this inoculum mixture was mixed with 20 kg maize seeds so that the seeds were coated by the inoculum mixture. For cropping cycle 1, the uncoated or AMF coated maize seeds ("Bisma" variety) were planted by dibbling 2 seeds per planting hole at 70 cm x 20 cm plant spacing. The entire dose of cattle manure (organic fertilizer) at different doses depending on the treatments (Table 1) was applied at the time of planting the maize seeds by burying the whole dose in the planting hole in the position below the seeds. The cattle manure variation in the fertilization package is to identify the optimum combinations to benefit the plant growth, increase the nutrient availability at the soils, and support the AMF development. The maximum combination of cattle manure was 15 ton/ha, the limit for transportation. The cattle manure applied in the package was measured with 3.08% Total Nitrogen, pH 6.66, 17.70 mg/kg of available P, 2.31 cmol/kg of available K, 10.45 C/N ration, and 32.2% C-organic. The inorganic (NPK) fertilizers with the recommended doses of 200 kg/ha Phonska (NPK 15:15:15) and 300 kg/ha Urea (46% N) were applied twice, i.e. at 7 DAS (days after seeding) and at 21 DAS, with the amount applied depending on the treatments (Table 1). At 7 DAS, the maize plants were fertilized with a mixture of the entire Phonska and one third of the Urea treatment doses, followed by application of the remaining Urea treatment doses at 21 DAS. The NPK fertilizers were applied in a furrow of 5 cm alongside the maize plant row at 5-7 cm depth before covered with soil.

After harvest of maize at 100 days after seeding (DAS) and field cleaning from maize debris, sorghum seeds were then planted in cropping cycle 2. Before the second sequence, the field was left fallow (rest) for 10 days. Seeds of sorghum (“Numbu” variety) were directly seeded by dibbling 2 seeds per planting holes made around the maize stubbles. These sorghum plants were not fertilized nor inoculated with additional AMF inoculum, but only the chopped maize roots containing the AMF mycorrhizal and spread throughout the plot. For both crops, the young maize and sorghum plants were tinned at 7 DAS by leaving one maize or sorghum plant per planting hole. Weeding and soil piling of the maize and sorghum plant base were done at 15 and 30 DAS. Plant protection was done by spraying “OrgaNeem” (an organic pesticide of plant origin containing Azadirachtin extracted from neem leaves) at a concentration of 5 ml OrgaNeem per liter of water. The OrgaNeem solution was applied to both crops (maize or sorghum) from 10 to 60 DAS at a 3-day interval. Harvesting of maize or sorghum crops was done at 100 DAS.

Measurement and Data Analysis

The variables measured were AMF development, N and P nutrition, and growth and yield of maize and sorghums. The AMF development includes spore counts at 60 and 100 DAS, and root colonization levels at 60 DAS. The N and P nutrition includes concentration of total N and available P in the rhizosphere of maize and sorghum at the time when the vegetative and generative growth found optimum, respectively at 60 and 100 DAS. The crop variables include dry weight (shoots and roots) and yield components (grain).

Laboratory analysis was conducted at Laboratory of Chemistry and Soil Science, Faculty of Agriculture, University of Mataram. Soil pH and texture were measured by standard procedures (Imam & Didar, 2005). Determination of total nitrogen in soil was done by destruction with $(NH_4)_2SO_4$ and distillation with NaOH where the $NH_4^+$was determined by indophenol blue colorimetric method and the NH_3 was defined by a titration with 0.05N of H_2SO_4 solution (Page et al., 1982). Total N in plants was measured using spectrophotometric indophenol blue methods with wave length 636 nm after destruction by $(NH_4)_2SO_4$ and distillation with NaOH following the Conway instruction (Lisle et al., 1990). The available Phosphorus in soil and plant was measured using spectrophotometer (λ = 693 nm) after the extraction process using Bray and Kurt I solution (0.025 N HCl + NH4F 0.03 N) (Bray & Kurtz, 1945). Total organic C was measured by oxidation with $K_2Cr_2O_7$ in presence of sulphuric acid (H_2SO_4) following Walkley and Black's method (Horwitz, 2000).

AMF spore extraction from soil (100 g soil sample) was done using the wet sieving and decanting technique (Brundrett et al., 1996). The spores collected from the 38 µm sieve after the final centrifugation were captured in a filter paper, which were then observed on a Petri dish using a stereo microscope at 40 x magnification, and counted as spore number per 100 g of soil. The percentage of root colonization was determined using the Gridline Intersect technique (Giovannetti & Mosse, 1980) under a stereo microscope after the root pieces were stained using the clearing and staining method of Brundrett et al. (1996).

Data were analyzed using analysis of two way ANOVA and the Tukey's HSD (Honestly Significant Difference) means tested at 5% level of significance.

RESULTS AND DISCUSSION

AMF Development

In this study, however, maize and sorghum were grown in sequence, in which sorghum was directly seeded without treatments and without tillage following the harvest of maize plants. All treatments, including AMF inoculation, were applied only to maize plants in the first cropping cycle. Therefore, it means that sorghum in cropping cycle 2 was grown after AMF propagules have built up in the soil during the growth of the maize plants in cropping cycle 1. This is in line with the results reported in the previous study (Arihara & Karasawa, 2000) which revealed that AMF root colonization and shoot dry weight of maize at 58 DAS were affected by the previous crop grown, whether they were host or non-host of AMF. When grown simultaneously, Carrenho et al. (2007) found higher colonization rates on maize than on sorghum. However, the P fertilization did not affect root colonization, especially on maize following AMF host plants.

Specifically, the degree of root colonization by AMF may be higher in the roots of maize fertilized with highly soluble mineral fertilizers (NPK, diammonium phosphate, ammonium sulfate and Urea). On the other hand, the length of extra-radical mycelium and number of AMF spores were significantly higher in maize fertilized with organic fertilizers (Bokashi manure) (Bautista-Cruz et al., 2014). This indicates that organic fertilization is more favorable for spore production compared with conventional fertilization. It can also be seen from Table 2 that AMF spore numbers are higher at harvest of the maize crop (100 DAS) compared with those at 60 DAS, especially on the D_2 treatment. This indicates a high buildup of AMF propagules for the

Table 2
Mean spore number (spores per 100 g soil) at 60 and 100 DAS, and colonization rates (%-colonization) on maize and sorghum in a maize-sorghum cropping sequence

Fertilization packages	AMF on maize (1st crop)			AMF sorghum (2nd crop)		
	Spore per 100 g soil		% colonization 60 DAS	Spore per 100 g soil		% colonization 60 DAS
	60 DAS	100 DAS		60 DAS	100 DAS	
D_0	764^{e}	3464^{d}	30^{d}	1231^{e}	3761^{d}	35^{e}
D_1	1059^{d}	3672^{c}	55^{c}	1343^{d}	4942^{b}	60^{d}
D_2	2119^{a}	4327^{a}	75^{a}	2981^{a}	5165^{a}	81^{a}
D_3	1690^{b}	3894^{b}	65^{b}	1881^{b}	4831^{c}	77^{b}
D_4	1294^{c}	3881^{b}	63^{b}	1769^{c}	4819^{c}	68^{c}
HSD 5%	231	13	2.0	109	12	6.5

Remarks: Mean values in each column followed by the same letters are not significantly different between treatments of fertilization packages; please refer to Table 1 for descriptions of the packages

subsequent sorghum crops. This condition seems to favor higher AMF colonization levels in roots of sorghum in the second cropping cycle compared with that of maize in the first cropping cycle. With no tillage treatment, the AMF colonization levels in sorghum may be higher than the levels in maize or bean roots (Alguacil et al., 2008). In addition, AMF colonization rates may be higher in roots of sorghum than in the roots of maize, either inoculated with *Glomus mosseae* or *Glomus versiforme*, indicating that sorghum is more favorable for AMF development than maize plants (Guo et al., 2013).

There are many factors influencing the degrees of AMF colonization of crop roots as reported by Carrenho et al. (2007). In examining the effects of different phosphorus, liming, organic matter, and soil texture on root colonization of peanuts, sorghum, and maize in a factorial experiment, each factor significantly affected root colonization level, alone or in interaction with other factors (Carrenho et al., 2007). The most surprising results were the significant interaction effects of plant, phosphorous, and organic matter although there were no significant effects of phosphorous (simple superphosphate of 166.6 mg/L (w/v) soil) nor organic matter (22 mg/L (w/v) soil). As for the interaction effect involving maize, there was no significant effect of phosphorous and organic matter on AMF colonization of maize roots. On the other hand, the application of both phosphorous and organic matter significantly reduced AMF colonization on sorghum roots (Carrenho et al., 2007).

In terms of fertilization effects, AMF colonization rate was reported to be higher on crop roots in unfertilized soils, followed by organic and biodynamic, and the least was seen in exclusively mineral fertilized and conventional farming systems. Thus, it was concluded that the amount of soluble P in the soil was the most limiting factor for AMF colonization in roots of those crops (winter wheat, vetch-rye, and grassclover) (Mäder et al., 2000). Moreover, it was reported that the increasing P level in the growing media from 0.1 to 0.5 mM P significantly reduced AMF colonization in roots of lettuce plants especially with increasing levels of N contents (1, 5, or 9 mM N) (Azcón et al., 2003).

A negative impact on soil condition occur when the accumulation of soil P has increased beyond the requirement of the crops cultivated (Grant et al., 2005). In the study, it seemed that the amount of P input from the NPK fertilizer as applied in the D_2 treatment was most favorable for AMF development in maize crops. In the D_2 treatment, the NPK fertilizer was reduced to 120 kg/ha Phonska (60% of the recommended NPK dose of 200 kg/ha Phonska in the D_0 treatment) combined with an application of 12 ton/ha cattle manure and inoculation with AMF. Among the treatments with AMF in combination with cattle manure, the D_1 treatment had the highest NPK fertilizer dose (Table 1), and this level of NPK fertilization seems to limit the AMF infection and development in maize roots compared with those in the D_2 treatment, resulting in a higher AMF colonization rate on D_2 than on D_1 treatment. Therefore, AMF colonization and spore numbers in maize were highest in the D_2 treatment, especially when compared with the D_1 or D_0 treatment. Using the same indigenous AMF applied to a pot experiment in which the soil for the growing media was taken from the same field in North Lombok, it was found that the highest level of AMF colonization of maize roots was in the treatment with AMF combined with cattle manure compared with AMF combined with NPK fertilizer or phosphate rock (Astiko et al., 2013a). The previous study also reported a significantly higher AMF spore number in plots fertilized with cattle manure or cattle manure combined with mineral fertilizers compared with those fertilized with mineral fertilizers only (Gryndler et al., 2006).

Soil Nutrient Status and Nutrient Sorption by Maize and Sorghum

There were significant effects of the different fertilizer packages on soil nutrient status (N and P contents of the soil) of the rhizosphere of both maize and sorghum crops at 60 and 100 DAS (Table 3). In general, there is a tendency that the highest values of the soil nutrient status, measured either at 60 or 100 DAS, are highest under the D_2 (60% NPK + 12 t/ha manure + AMF) or D_1 (80% NPK + 15 t/ha manure + AMF) treatment. The values of the soil nutrient status in these treatments were higher than those in D_0 (100% NPK only) treatment, although the dose of NPK fertilizers was reduced in the D_1 and D_2 treatments (Table 3).

At the present study, no infiltration data was measured, however it is important to note that since the NPK fertilizers applications, i.e. Phonska and Urea at 7 DAS and Urea at 21 DAS, at sandy lands, are easily dissolved in water, and significant amount of the nutrients could have been loss through infiltration during the rainy season. Previous study shows that the sand content of the cultivated land had a positive correlation with infiltration rate, with an

Table 3
Mean concentration of total N and available P of soil in the rhizospheres of maize (1st crop) and sorghum (2nd crop) for each treatment of fertilization packages, measured at 60 and 100 DAS

Fertilization packages	Total N ($g.kg^{-1}$) at 60 DAS		Available P ($mg.kg^{-1}$) at 60 DAS		Total N ($g.kg^{-1}$) at 100 DAS		Available P ($mg.kg^{-1}$) at 100 DAS	
	maize	sorghum	maize	sorghum	maize	sorghum	maize	sorghum
D_0	1.24^{c}	1.10^{c}	14.97^{d}	11.41^{d}	1.33^{d}	1.23^{d}	17.43^{d}	10.15^{d}
D_1	1.45^{a}	1.29^{a}	28.44^{b}	16.25^{b}	1.69^{b}	1.38^{b}	29.51^{b}	21.58^{b}
D_2	1.47^{a}	1.25^{ab}	35.02^{a}	28.49^{a}	1.86^{a}	1.48^{a}	36.56^{a}	29.99^{a}
D_3	1.39^{b}	1.22^{ab}	17.52^{c}	14.59^{bc}	1.47^{c}	1.31^{c}	19.37^{c}	18.59^{c}
D_4	1.33^{b}	1.20^{b}	16.29^{c}	14.25^{c}	1.42^{c}	1.31^{c}	18.53^{c}	17.32^{c}
HSD 5%	0.08	0.07	1.31	1.99	0.08	0.06	1.0	2.98

Remarks: Mean values in each column followed by the same letters are not significantly different between treatments of fertilization packages; please refer to Table 1 for description of the packages

r-value of +0.75 (Patle et al., 2018). Since cattle manure is slow in releasing nutrients, it is possible that at the time of measurement (60 and 100 DAS), the loss of released nutrients from the NPK fertilizers through leaching was much higher in the D_0 than in the other treatments of fertilization packages due to dissolution by rain water during the rainy season. However, there were small increases in nutrient status of the soil from 60 to 100 DAS around the rhizospheres of maize crop, especially in those treated with manure and AMF application. This indicated a slow release of N and P nutrients from manure after application to the maize plants in the first cropping cycle, which could be due to AMF inoculation. Many studies have reported that AMF can mobilize and take N and P from organic matters for their hosts (Feng et al., 2003; Hawkins et al., 2000; Koide & Kabir, 2000; Leigh et al., 2009; Tarafdar & Marschner, 1994), and it can be seen from Table 2 that the highest average of AMF colonization levels in maize roots was in the D_2 treatment. Therefore, N and P uptake in shoots of maize and sorghum was highest in the D_2 treatment (Table 4).

In addition, soil contents of those nutrients corresponded well with the levels of N and P uptake of the crops, i.e. all showing positive correlation coefficients. Although it is not significant for P uptake, the highest values of N and P uptake were also seen in the D_2 treatment for both maize and sorghum (Table 4). The correlation analysis of the mean values obtained at 60 DAS showed a significant correlation between soil N and N sorption in the shoots, with a value of r = + 0.970 (R-square = 94.1%, p = 0.006) for maize plants, and r = + 0.904 (R-square = 81.7%, p = 0.035) for sorghum plants. These indicate those fertilizers in the packages contribute to nutrient contents of the crops, which are significantly different between treatments of fertilization packages (Table 4).

However, P status of the soil did not show a significant correlation with P uptake either by maize or sorghum crop. In spite of that, there is a significant positive correlation

Table 4
Mean N and P sorption (mg.g^{-1} plant dry weight) by each crop at 60 DAS for each treatment of fertilization packages

Fertilization packages	N and P uptake (mg.g^{-1} plant dry weight) by each crop at 60 DAS			
	Maize (1st cropping cycle)		Sorghum (2nd cropping cycle)	
	N	P	N	P
D_0	18.62^e	1.71^d	12.74^d	0.65^d
D_1	27.58^b	2.60^b	18.92^b	0.85^c
D_2	28.00^a	2.72^a	20.86^a	1.48^a
D_3	23.10^c	2.41^c	17.29^c	1.36^b
D_4	20.44^d	2.41^c	17.22^c	1.32^b
HSD 5%	0.41	0.11	0.07	0.04

Remarks: Mean values in each column followed by the same letters are not significantly different between treatments of fertilization packages; please refer to Table 1 for description of the packages

between the degree of AMF colonization in the roots and P sorption in the shoot of maize plants, with an r = + 0.909 (R-square = 82.6%, p = 0.032), and that of sorghum plants, with an r = + 0.940 (R-square = 88.4%, p = 0.017). This shows that there were significant contributions of AMF colonization in the roots to the amount of P taken up by the plants, both maize and sorghum. If soil soluble P has not exceeded crop requirements, AMF association will still result in significantly positive effects on P uptake and biomass production because of P requirements of the crops; for optimum growth and yields, crops require P since the early stage of their growth, either from soil, fertilizer application, or from AMF associations (Grant et al., 2005).

Although sorghum crop in the second cropping cycle was not fertilized with manure nor NPK fertilizers, direct seeding of sorghum after harvesting the maize plants without tillage seemed to be a favorable condition in establishing AMF association in the sorghum crop, which resulted in higher AMF colonization rates in sorghum than in maize roots for each fertilizer package (Table 2). The AMF associations seemed to enable the sorghum crops to absorb adequate P and other nutrients from soil and residues of the manure applied in the first cropping cycle even though these sorghum crops were not fertilized. This could occur because of the potential of AMF external hyphae that help the host plants in absorbing and dissolving nutrients from soil and manures and other nutrient pools, as well as other nutrients unavailable for uptake by plant roots (Menge, 1983; Smith & Read, 2010; Tawaraya et al., 2006). Guo et al. (2013) also reported significant effects of AMF inoculation on C, N, P, and K contents (mg/pot) in shoot and roots of maize and sorghum grown in pots filled with mixtures of mine tailing and topsoil, and between the AMF species used, *G. versiforme* showed higher colonization rates which resulted in higher nutrient sorption and biomass of maize and sorghum compared with *G. mosseae* (Guo et al., 2013).

Biomass and Yield Components of Maize and Sorghum

In terms of biomass production and yield components of maize and sorghum, there were also significant effects of the fertilization packages in which D_2 treatment presents the highest values of biomass production (Table 5) and yield components (Table 6). This means that the nutrient status of the soils corresponded well with the biomass weight of the crops, indicated by the positive correlation coefficients, although only some of them were significant. The AMF applications to maize crops, observed at 60 DAS, showed a significant correlation to dry shoot weight, N and P status of the soil, and the N and P sorption. In contrast, AMF colonization levels of maize roots only showed significant correlation with root dry weight at 60 DAS, with an r = + 0.912 (R^2 = 83.2%, p= 0.031) and shoot dry weight at maturity (100 DAS), with an r = + 0.892 (R^2 = 79.6%, p= 0.042). This could mean that during the vegetative growth, AMF colonization have focused on improving root growth to increase nutrient sorption during the vegetative growth of maize crops.

In relation to nutrient uptake, although AMF colonization levels did not show significant correlation with N uptake in shoots of maize or sorghum, they did show a positive significant correlation with P uptake both in shoots of maize and sorghum, with an r = + 0.909 (R^2 = 82.6%, p = 0.032) for maize, and r = + 0.940 (R^2 = 88.4%, p = 0.017) for sorghum. These could be due to the ability of AMF extra-radical hyphae in mobilizing and absorbing P from organic matters for their hosts (Feng et al., 2003; Koide & Kabir, 2000; Tarafdar & Marschner, 1994). Based on the strength of the relationships between AMF colonization levels and P uptake, the value of R^2 is higher in sorghum (R^2 = 88.4%) than in maize (R^2 = 82.6%). This means that the contribution of AMF colonization in roots to P uptake in shoots was higher in sorghum crop at the second cropping cycle than in maize in the first cropping cycle. Since sorghum did not receive any fertilizers, and cattle manure is a

Table 5
Mean dry biomass weight (g/plant) of maize and sorghum for each treatment of fertilization packages

Fertilization packages	Dry biomass weights (g/plant) of maize (1st crop) and sorghum (2nd crop)							
	Maize root		Maize shoot		Sorghum root		Sorghum shoot	
	60 DAS	100 DAS	60 DAS	100 DAS	60 DAS	100 DAS	60 DAS	100 DAS
D_0	8.13^d	10.43^e	34.15^d	62.29^e	0.82^d	12.30^d	8.31^d	47.74^e
D_1	15.13^b	22.39^b	61.92^b	109.03^b	2.22^b	22.34^b	16.89^b	95.01^b
D_2	17.12^a	34.56^a	72.86^a	111.25^a	3.37^a	24.44^a	23.53^a	105.14^a
D_3	13.65^c	18.48^c	52.26^c	101.05^c	1.68^c	14.52^c	12.01^c	85.46^c
D_4	13.34^c	15.43^d	51.29^c	95.87^d	1.38^c	14.47^c	11.81^c	66.06^d
HSD 5%	0.31	3.04	0.97	2.21	0.30	0.05	0.20	9.54

Remarks: Mean values in each column followed by the same letters are not significantly different between treatments of fertilization packages; please refer to Table 1 for description of the packages slow-release organic fertilizer, it could mean that most P nutrient contained in the shoots, especially in shoots of sorghum in the second cropping cycle, was mostly taken up from the manure under the contribution of AMF colonization in the roots. However, this still needs to be confirmed by further research. This view is supported by the conditions of the study area, which was dominated by sand, and if leaching happened during the rainy season, the loss of dissolved nutrients from the NPK fertilizers applied in the first cropping cycle could be high. Therefore, the main source of nutrients, especially for sorghum crop in the second cropping cycle was the cattle manure applied to the maize crop in the first cropping cycle.

In relation to nitrogen nutrient, there was a very low and non-significant correlation between AMF colonization levels and N uptake in maize shoots with an R^2 of only 41.2%. However, the N uptake of maize at 60 DAS was highly significantly correlated with the N status of the soil with an R^2 = 94.1% (p = 0.006). Moreover, the N soil availability also showed a significant correlation with biomass and grain yield of maize, i.e. with an R^2 = 84.1% (p = 0.029) with grain yield at 60 DAS, R^2 = 92.2% (p = 0.009) with shoot dry weight at 60 DAS, and R^2 = 90.6% (p = 0.012) with shoot dry weight at 100 DAS.

In contrast, for sorghum in the second cropping cycle, the soil N status at 60 DAS showed a significant correlation only with shoot dry weight at 100 DAS. Unlike in maize crop where AMF colonization levels showed a significant and positive correlation with shoot dry weight at maturity (100 DAS), AMF colonization levels in sorghum roots did not show any significant correlation with biomass weight or yield components of sorghum. However, AMF colonization rates in sorghum roots showed a sufficiently high correlation with N uptake in sorghum shoots, with an R^2 = 71.9% (p = 0.069), and N uptake in sorghum shoots showed positive significant correlation with biomass and grain yield of sorghum, i.e. with an R^2 = 80.5% (p = 0.039) with grain

Table 6
Mean weights of total dry grains (kg/plot) and 100 dry grains for each crop and each treatment of fertilization packages

Fertilization packages	Mean dry grain yield (kg/plot) and weight of 100 dry grains (g)			
	Maize in the 1st cropping cycle		Sorghum in the 2nd cropping cycle	
	Grain yield	100 grains	Grain yield	100 grains
D_0	9.60e	22.48d	3.57d	2.73d
D_1	17.40b	26.94b	5.05b	3.01b
D_2	22.80a	28.12a	6.65a	3.61a
D_3	15.60c	25.98c	4.43c	2.90c
D_4	10.20d	24.61c	4.17c	2.81cd
HSD 5%	0.59	1.37	0.26	0.09

Remarks: Mean values in each column followed by the same letters are not significantly different between treatments of fertilization packages; please refer to Table 1 for description of the packages yield, R^2 = 83.0% (p = 0.031) with shoot dry weight at 60 DAS and R^2 = 89.9% (p = 0.014) with shoot dry weight at 100 DAS. No additional fertilizers and inoculums were applied at the cropping cycle 2. These could mean that for maize crop, most nutrients were derived from NPK fertilizers while for sorghum, N and P nutrients were derived from the residues of manure contributed from AMF colonization in sorghum roots. Many researchers have also shown the ability of AMF to utilize organic sources to supply N to their host (e.g. Hawkins et al., 2000; Leigh et al., 2009) although it was also found that AMF colonization did not necessarily result in significantly higher N status of the mycorrhizal than non-mycorrhizal hosts (Hawkins et al., 2000).

However, when the correlation analysis was done between averages of colonization levels, biomass and grain yield between maize in the first and sorghum in the second cropping cycle, in general the results show significant and positive coefficients of correlation. For example, correlation of AMF colonization levels between roots of maize and sorghum are highly significant, with an r = + 0.988 (R^2 = 97.6%, p = 0.002). This means that the pattern of differences in AMF colonization levels between roots of maize and sorghum is highly similar. In other words, AMF colonization levels in roots of maize in the first cropping cycle were carried over into the second cropping cycle where sorghum was grown as the rotation crop. This is because both maize and sorghum are hosts of AMF, and both crops have high mycorrhizal dependency (Guo et al., 2013).

In addition, when a t-test of ‘Paired Two Sample for Means’ was run on the averages of AMF colonization levels and spore number in soil at maturity of the crops between maize and sorghum in Table 2, higher significant values (p<0.01) were seen on sorghum than on maize. This could be due to the buildup of AMF in the soil after maize crop in the first copping cycle was harvested before sorghum was directly seeded without tillage. Even though both crops were grown simultaneously, it was also found that AMF colonization levels were higher on sorghum than on maize (Guo et al., 2013). This means that sorghum can be used to improve mycorrhizal conditions of the soil under a maize-sorghum cropping sequence.

CONCLUSION

Among the mycorrhiza-based fertilization packages tested on the maize-sorghum cropping sequence system at the drylands in North Lombok of Indonesia, the D_2 package, consisting of 60% NPK recommended dose, 12 t/ha cattle manure, and the indigenous AMF inoculation, was found to be the best fertilization package to improve crop yield and soil nutrient availability. This study noted that the AMF development was higher in the sorghum at the second cropping cycle compared to the growth in the maize at the first cropping cycle. This condition led to the higher NP-uptake and soil nutrient availability in subsequent crops, both at the sandy dryland, with no additional fertilization and mycorrhizal propagules applied.

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REFERENCES

Alguacil, M., Lumini, E., Roldan, A., Salinas-Garcia, J., Bonfante, P., & Bianciotto, V. (2008). The impact of tillage practices on arbuscular mycorrhizal fungal diversity in subtropical crops. *Ecological Applications*, *18*(2), 527-536.

Arihara, J., & Karasawa, T. (2000). Effect of previous crops on arbuscular mycorrhizal formation and growth of succeeding maize. *Soil Science and Plant Nutrition*, *46*(1), 43-51.

Astiko, W. (2013). *The role of indigenous mycorrhizae in different plant patterns to increase soybean yield in sandy soil (Case studies on dryland North Lombok)* (Unpublished Doctoral thesis), University of Brawijaya, Indonesia.

Astiko, W., Sastrahidayat, I. R., Djauhari, S., & Muhibuddin, A. (2013a). The role of indigenous mycorrhiza in combination with cattle manure in improving maize yield (*Zea mays* L.) on sandy loam of Northern Lombok, Eastern of Indonesia. *Journal of Tropical Soils*, *18*(1), 53-58.

Astiko, W., Sastrahidayat, I. R., Djauhari, S., & Muhibuddin, A. (2013b). Soil fertility status and soybean [*Glycine max* (L.) Merr.] performance following introduction of indigenous mycorrhiza combined with various nutrient sources into sandy soil. *AGRIVITA, Journal of Agricultural Science*, *35*(2), 127-137.

Augé, R. M. (2004). Arbuscular mycorrhizae and soil/plant water relations. *Canadian Journal of Soil Science*, *84*(4), 373-381.

Azcón, R., Ambrosano, E., & Charest, C. (2003). Nutrient acquisition in mycorrhizal lettuce plants under different phosphorus and nitrogen concentration. *Plant Science*, *165*(5), 1137-1145.

Bautista-Cruz, A., Cruz-Domínguez, G., Rodríguez-Mendoza, M. d. l. N., Pérez-Pacheco, R., & Robles, C. (2014). Effect of compost and slow-release fertilizers addition on soil biochemistry and yield of maize (*Zea mays* L.) in Oaxaca, Mexico. *Revista de la Facultad de Ciencias Agrarias*, *46*(1), 181-193.

Bray, R. H., & Kurtz, L. T. (1945). Determination of total, organic, and available forms of phosphorus in soils. *Soil Science*, *59*(1), 39-46.

Bruand, A., Hartmann, C., & Lesturgez, G. (2005). *Physical properties of tropical sandy soils: A large range of behaviours*. Retrieved February 26, 2019, from https://hal-insu.archives-ouvertes.fr/file/index/docid/79666/filename/Bruand_keynote_paper.pdf

Brundrett, M., Bougher, N., Dell, B., & Grove, T. (1996). *Examining mycorrhizal associations*. Retrieved February 26, 2019, from http://irt-pw-cp1.irt.csus.edu/jbaxter/wp-content/uploads/2016/02/1996-Brundrett-et-al-Mycorrhizal-Methods-Book.pdf

Calderón-Vázquez, C., Alatorre-Cobos, F., Simpson-Williamson, J., & Herrera-Estrella, L. (2009). Maize under phosphate limitation. In *Handbook of maize: Its biology* (pp. 381-404). New York, NY: Springer.

Carrenho, R., Trufem, S. F. B., Bononi, V. L. R., & Silva, E. S. (2007). The effect of different soil properties on arbuscular mycorrhizal colonization of peanuts, sorghum and maize. *Acta Botanica Brasilica*, *21*(3), 723-730.

Cavagnaro, T. R., Bender, S. F., Asghari, H. R., & van der Heijden, M. G. (2015). The role of arbuscular mycorrhizas in reducing soil nutrient loss. *Trends in Plant Science*, *20*(5), 283-290.

Dhillion, S. S., & Ampornpan, L. A. (1992). The influence of inorganic nutrient fertilization on the growth, nutrient composition and vesicular-arbuscular mycorrhizal colonization of pretransplant rice (*Oryza sativa* L.) plants. *Biology and Fertility of Soils*, *13*(2), 85-91.

Drew, E., Murray, R., Smith, S., & Jakobsen, I. (2003). Beyond the rhizosphere: Growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. *Plant and Soil*, *251*(1), 105-114.

Feng, G., Song, Y., Li, X., & Christie, P. (2003). Contribution of arbuscular mycorrhizal fungi to utilization of organic sources of phosphorus by red clover in a calcareous soil. *Applied Soil Ecology*, *22*(2), 139-148.

George, E., Marschner, H., & Jakobsen, I. (1995). Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. *Critical Reviews in Biotechnology*, *15*(3-4), 257-270.

Giovannetti, M., & Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist*, *84*(3), 489-500.

Grant, C., Bittman, S., Montreal, M., Plenchette, C., & Morel, C. (2005). Soil and fertilizer phosphorus: Effects on plant P supply and mycorrhizal development. *Canadian Journal of Plant Science*, *85*(1), 3-14.

Gryndler, M., Larsen, J., Hršelová, H., Řezáčová, V., Gryndlerová, H., & Kubát, J. (2006). Organic and mineral fertilization, respectively, increase and decrease the development of external mycelium of arbuscular mycorrhizal fungi in a long-term field experiment. *Mycorrhiza*, *16*(3), 159-166.

Guo, W., Zhao, R., Zhao, W., Fu, R., Guo, J., Bi, N., & Zhang, J. (2013). Effects of arbuscular mycorrhizal fungi on maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) grown in rare earth elements of mine tailings. *Applied Soil Ecology*, *72*, 85-92.

Hawkins, H. J., Johansen, A., & George, E. (2000). Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil*, *226*(2), 275-285.

Horwitz, W. (2000). *Official methods of analysis of AOAC International* (17th ed., Vol. 1). Gaithersburg, USA: AOAC International.

Imam, S. M. H., & Didar, M. A. (Eds.) (2005). *A handbook on analysis of soil, plant and water*. Dhaka, Bangladesh: Bangladesh-Australia Centre for Environmental Research (BACER-DU).

Johnson, N. C., Copeland, P. J., Crookston, R. K., & Pfleger, F. (1992). Mycorrhizae: Possible explanation for yield decline with continuous corn and soybean. *Agronomy Journal*, *84*(3), 387-390.

Koide, R., & Kabir, Z. (2000). Extraradical hyphae of the mycorrhizal fungus *Glomus* intraradices can hydrolyse organic phosphate. *New Phytologist*, *148*(3), 511-517.

Leigh, J., Hodge, A., & Fitter, A. H. (2009). Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytologist*, *181*(1), 199-207.

Lisle, L., Gaudron, J., & Lefroy, R. (1990). *Laboratory techniques for plant and soil analysis*. Armidale, Australia: UNE-ACIAR- Crawford Fund.

Mäder, P., Edenhofer, S., Boller, T., Wiemken, A., & Niggli, U. (2000). Arbuscular mycorrhizae in a long-term field trial comparing low-input (organic, biological) and high-input (conventional) farming systems in a crop rotation. *Biology and Fertility of Soils*, *31*(2), 150-156.

Marulanda, A., Azcon, R., & Ruiz-Lozano, J. M. (2003). Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by Lactuca sativa plants under drought stress. *Physiologia Plantarum*, *119*(4), 526-533.

Menge, J. (1983). Utilization of vesicular–arbuscular mycorrhizal fungi in agriculture. *Canadian Journal of Botany*, *61*(3), 1015-1024.

Page, A. L., Miller, R. H., & Keeney, D. R. (1982). *Methods of soil analysis, Part 2: Chemical and microbiological properties* (2nd Ed.). Madison, USA: American Society of Agronomy.

Patle, G. T., Sikar, T. T., Rawat, K. S., & Singh, S. K. (2018). Estimation of infiltration rate from soil properties using regression model for cultivated land. *Geology, Ecology, and Landscapes*, *3*(1), 1-13.

Plenchette, C., Fortin, J., & Furlan, V. (1983). Growth responses of several plant species to mycorrhizae in a soil of moderate P-fertility. *Plant and Soil*, *70*(2), 199-209.

Smith, S. E., & Read, D. J. (2010). *Mycorrhizal symbiosis*. New York, NY: Academic Press.

Supardi, G. (1996). Mining the synergistic effects towards strong agriculture. *HITI News*, *4*, 10-13.

Tarafdar, J., & Marschner, H. (1994). Phosphatase activity in the rhizosphere and hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. *Soil Biology and Biochemistry*, *26*(3), 387-395.

Tawaraya, K. (2003). Arbuscular mycorrhizal dependency of different plant species and cultivars. *Soil Science and Plant Nutrition*, *49*(5), 655-668.

Tawaraya, K., Naito, M., & Wagatsuma, T. (2006). Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. *Journal of Plant Nutrition*, *29*(4), 657-665.

Vivekanandan, M., & Fixen, P. (1991). Cropping systems effects on mycorrhizal colonization, early growth, and phosphorus uptake of corn. *Soil Science Society of America Journal*, *55*(1), 136-140.

Wangiyana, W., Cornish, P. S., & Morris, E. (2006). Arbuscular mycorrhizal fungi dynamics in contrasting cropping systems on vertisol and regosol soils of Lombok, Indonesia. *Experimental Agriculture*, *42*(4), 427-439.

Zhu, Y. G., Cavagnaro, T. R., Smith, S. E., & Dickson, S. (2001). Backseat driving? Accessing phosphate beyond the rhizosphere-depletion zone. *Trends in Plant Science*, *6*(5), 194-195.

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Agro-Morphological Characterization "*In situ*" of *Tamarindus indica* L. in the Dry Forest of Ecuador

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ABSTRACT

Tamarind (*Tamarindus indica* L.) is a species of high economic potential in local markets in Ecuador. This species is mainly used for its nutritional, industrial, medicinal and/or therapeutic attributes, in addition to its use as an ornamental tree. However, scarce information has been generated about this little explored species. In order to identify individuals with outstanding quality and production characteristics, an *in situ* morphological characterization of 32 individuals was carried out in the provinces of Manabí, Guayas and Loja. Qualitative and quantitative traits were evaluated using descriptive, parametric and multivariate analysis. The date of flowering and harvesting were determined as qualitative discriminant variables; while the discriminant quantitative traits were plant height, seed number per pod, pulp percentage, peel percentage and fruit-rib percentage. Cluster analysis established six groups; however, little variability was observed based on the morphological genetic distance, thus it could indicate that there is a low genetic abundance of *T. indica* in the dry forest of Ecuador.

Keywords: Accessions, breeding, descriptors, Fabaceae, tamarind

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INTRODUCTION

There are more than 250 species of tropical fruits worldwide, nevertheless only a few of them are commercially exploded, due to the little research, lack of promotion and alternatives for their trade (Cruz & Deras, 2000). *Tamarindus indica* L. (Fabaceae/Caesalpiniodeae), is a fruit tree native of

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tropical Africa, and now distributed in several tropical regions in semi desert conditions, with restrictions to low temperature (Tapia et al., 2012). Despite all of this, the origin of tamarind is still a controversy (Diallo et al., 2008). Considered as the "king of the fruits" by the Sakalava people of Madagascar (Ballarin & Raison, 2000), the main product of tamarind is the pulp of the fruit, which has an acid or sweet flavor and it is used in industrial and traditional meals in Asia, Africa and America (Zetina et al., 2012), feed for livestock and medicines (Caluwe et al., 2010; El-Siddig et al., 2006; Graf et al., 2016).

Tamarind has an important role in local economies (El-Siddig et al., 2006). The main producers of this fruit are India (300,000 tons) and Thailand (140,000 tons). On the other hand, in the American continent, the production is found mainly in México (37,820 tons) and Costa Rica (220 tons) (Zetina et al., 2012).

In Ecuador, this species is present in several provinces of the pacific coast in elevations from 0 to 1,500 meters above sea level, in zones with low rainfall, high luminosity and low nutritional soils; however, tamarind is cultivated in disperse ways such as backyards and other land uses as part of urban landscape instead of commercial level plantation exploitations.

The propagation of tamarind in Ecuador has been mainly by seed, without a selection process. On the other hand, farmers and forestry programs have contributed to the degradation of the species, by establishing new growths using trees with unknown origins and possibly with undesirable phenotypics characters. Nowadays countries such as Mexico have put interest in this fruit tree in subjects like genetic erosion, plant breeding and the selection of population *in situ* (Fandohan et al., 2010), this last methodology allowing the identification of plus or elite trees with outstanding characters in yield and quality with the objective of future incorporation in plant breeding programs (Zetina et al., 2012).

According to Sarmiento et al. (2017), Ecuadorian tamarind genotypes have showed a reasonable degree of genetic diversity that can be used as basis for hybridization. Consequently, the objective of this study was to identify outstanding tamarind individuals under *in situ* conditions by the morphological characterization of 32 accessions of this fruit tree recollected in three provinces of Ecuador and to establish a baseline for the genetic breeding of this species. In addition, morphological variability of this species (32%) has been reported in the dry forest of Colombia (Álvarez et al., 2018). For this reason, this study evaluated different accessions of Tamarind located in the Ecuadorian dry forest, in order to perform a morphological characterization and identify elite plant traits.

MATERIALS AND METHODS

This research was carried out between January and December of 2015 in the coastal provinces of Manabí, Guayas and Loja; 32 farms were visited, corresponding to 22 sites and nine cantons (Table 1).

Each farm was georeferenced and once the tamarind population was identified, the best individual of that population was selected on the basis of information provided by the owner of the farm, in relation to the alternation of the harvest, productivity, phytosanitary status, vigor in the productive stage and presence of fruits of high quality. A total of 32 individuals (accessions) were selected based on the above criteria.

Table 1
Geographic location of each in situ selected tamarind tree

Accession code	Location			Coordinates		Altitude (masl)
	Site	County	Province	Longitude	Latitude	
TI-ECUM-001	Joa	Jipijapa	Manabí	26°36´2,0"	01°05´49,1"	
TI-ECUM-002				26°36´2,0"	01°05´48,7"	79.3
TI-ECUM-003				26°36´1,6"	01°05´48,3"	
TI-ECUM-004	Cantagallo		Manabí	80°48´27,3"	01°17´01,2"	105.8
TI-ECUM-005				80°43´27,8"	01°17´00,7"	
TI-ECUM-006	El Cady		Manabí	80°24´19,6"	01°07´04,5"	62.8
TI-ECUM-007	Maconta	Portoviejo	Manabí	80°21´16,6"	01°02´17,8"	83.2
TI-ECUM-008				80°21´16,8"	01°02´19,4"	
TI-ECUM-009	Tabacales	Rocafuerte	Manabí	80°26´43,2"	00°56´32,2"	36.6
TI-ECUM-010	Valdez		Manabí	80°26´31,8"	00°56´52,6"	45.4
TI-ECUM-011	El Cardón		Manabí	80°23´62,5"	00°54´55,7"	44.2
TI-ECUM-012	La Balsita		Manabí	80°23´38,5"	01°00´25,8"	44.8
TI-ECUM-013	La Horma		Manabí	80°23´44,9"	00°54´41,2"	78.3
TI-ECUM-014	Las Flores		Manabí	80°21´22,4"	00°55´37,5"	104.9
TI-ECUM-015	Zapatón		Manabí	80°28´22,9"	00°53´14,7"	21.0
TI-ECUM-016				80°28´22,5"	00°53´14,3"	24.9
TI-ECUM-017	Cristo Rey	Sucre	Manabí	80°29´39,1"	00°49´05,4"	35.9
TI-ECUM-018	El Blanco		Manabí	80°29´45,9"	00°49´02,1"	26.5
TI-ECUM-019	Costa Rica	Portoviejo	Manabí	80°27´43,0"	00°59´54,8"	28.4
TI-ECUM-020	El Retiro		Manabí	80°28´37,5"	00°59´31,6"	39.6
TI-ECUM-021	Lodana	Santa Ana	Manabí	80°23´16,8"	01°10´13,6"	73.2
TI-ECUM-022			Manabí	80°38´3,99"	01°19´96,6"	65.5
TI-ECUM-023				80°38´3,54"	01°19´96,2"	
TI-ECUM-024	Mate		Manabí	80°33´23,0"	01°22´87,5"	96.0
TI-ECUM-025	Los Tillales	24 de Mayo	Manabí	80°25´3,39"	01°15´0,86"	113.7
TI-ECUM-026	El Guarango	Rocafuerte	Manabí	80°24´14,3"	00°53´4,39"	43.3
TI-ECUG-027	Valle de la Virgen	Pedro Carbo	Guayas	80°11´46,2"	01°44´33,9"	77.4
TI-ECUM-028	Guale	Paján	Manabí	80°12´29,3"	01°40´50,4"	110.3
TI-ECUL-029	Garza Real	Zapotillo	Loja	80°13´58,3"	04°18´24,3"	236.0
TI-ECUL-030	Garza Real	Zapotillo	Loja	80°13´58,0"	04°18´24,0"	236.0
TI-ECUL-031	Garza Real	Zapotillo	Loja	80°13´57,7"	04°17´58,5	233.0
TI-ECUL-032	Garza Real	Zapotillo	Loja	80°13´17,1"	04°17´59,6"	232.0

The descriptors used were established based on scientific literature (Fandohan et al., 2011; International Plant Genetic Resources Institute [IPGRI], 1980; Zetina et al., 2012). Tamarind individuals were evaluated on six qualitative traits (vigor, harvest alternation, cup shape, pod type, flowering date and harvest date), and 14 quantitative variables (plant age, plant height, trunk diameter, seed/pod number, number of fruit/bunch, pod length, pod diameter, pulp/seed ratio, pulp percentage, shell percentage, seed percentage, nervure percentage, pulp + seed yield and fresh fruit yield). For the variables percentage of pulp, percentage of shell, percentage of seed and percentage of nervure, a random sample of 25 mature fruits was taken; the parts of the fruit were separated, weighed and the percentage of all components estimated.

Qualitative traits were analyzed using Chi square test to determine statistical differences, Cramer and Pearson coefficients were estimated. Quantitative variables were analyzed using descriptive statistics (frequency, mean range and coefficient of variation). In order to determine the structure of the clusters, we used multivariate statistics such as cluster analysis (Ward algorithm) using the minimum variance method. The quantitative variables inside the groups were evaluated by analysis of variance and comparison of means by the Duncan test. Analyzes were performed using the statistical package INFOSTAT version 1.1.

RESULTS AND DISCUSION

Of the selected individuals, 84% came from the province of Manabí, 13% from the province of Loja and 3% from the province of Guayas. Generally, farms own few tamarind trees and the majority were dispersed, predominating mainly in systems of home orchards or forming small units of production.

Qualitative Traits

In the 6 qualitative traits, Chi square test indicated statistical significance (P> 0.01) for the discriminant variables: flowering date and harvest date (Table 2), which would indicate that there are variability in these characters and possibly respond

Table 2
Chi square values, Cramer and Pearson contingency coefficients obtained in the qualitative traits of 32 individuals of tamarind selected in situ

Descriptor	Chi square	Cramer coeficient	Pearson coeficient	P value
Vigor	1.75	0.23	0.23	0.4169
Alternation	4.00	0.35	0.33	0.1353
Top shape	10.94	0.58	0.50	0.0042
Type of sheath	6.13	0.44	0.40	0.0133
Flowering date[D]	44.50	1.18	0.76	<0.0001**
Harvest date[D]	31.00	0.98	0.70	<0.0001**

[D] Discriminant traits
** Significative at 1%

to the prevailing conditions of each zone, where out-of-season rainfall or irrigation immediately after harvest would mark the phenological rhythms. The higher values of Cramer coefficient (1.18 and 0.98) and Pearson coefficient (0.76 and 0.70) for these two variables confirm their discriminant character.

Quantitative Traits

Of the selected trees, 56% were between 10 and 22 years of age, followed by 25% between the ages of 23 and 35, and the most adult individuals (> 36 years) accounted for 19% (Figure 1). This indicates that most of the individuals under study would not be in their full productive age, due to the life cycle of tamarind can range from 20 to 80 years (Troup, 1921). Generally, farmers do not usually prune the tamarind trees. Tree height was concentrated in its highest percentage (43%) between 9 and 12 m; while 28% of individuals reached a height between 5 and 8 m (Figure 1). Zetina et al. (2012) established that a suitable height to carry out an optimal management of this fruit tree would be of 2.5 to 3 m with pruning and giving the shape to the tree top to have lateral branches where the production would be generated. The individuals analyzed exceed this height notably because there was no pruning or other activity to reduce the top. Some observed individuals (6%) even reached heights of 17 to 20 m. In addition, this height condition makes harvesting and phytosanitary management of trees difficult. In several tropical fruit trees, pruning has been shown to reduce the tree height and diameter by 10 to 14% (Vásquez et al., 2009). In terms of tamarind, pruning is not a common practice and knowledge about its productive and physiological function (unknown by the farmers) would be useful to be more efficient in the use of the resources (water and nutrients) by the plant, especially in dry forest conditions. Most of the selected trees (85%) had diameters smaller than 41 cm; the remaining 15% of the individuals had diameters between 42 and 71 cm (Figure 1), a characteristic associated with the age of the plant because the trunk increases its thickness and vigorosity as the years advance (Zetina et al., 2012).

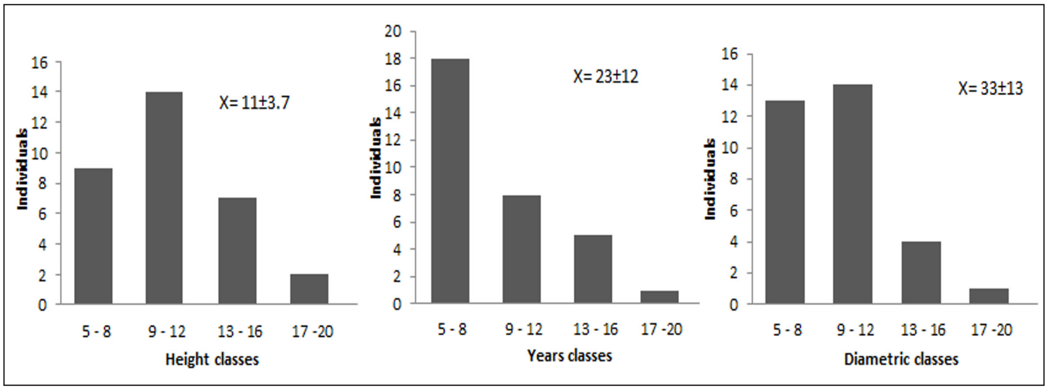

Figure 1. Frequency analysis for the variables plant age, plant height and stem diameter of the 32 individuals of tamarind selected *in situ*

Descriptive statistics of the quantitative traits are presented in Table 3. Of the fruit components, pulp represented 48.5%, which indicates that the accessions evaluated have a good productivity potential. In general, the 32 individuals had a percentage of pulp which ranged from 40 to 59%. Two trees (TI-ECUM-003 and TI-ECUM-004) from Jipijapa (Joa and Cantagallo sites) did not present pulp midrib; however, in other individuals when this trait was present, values were between 1 and 2 %. The evaluated individuals showed heterogeneity in the pod length, being observed a range of 7 to 13 cm, where 17 individuals (53%) obtained values greater than 10 cm, which is considered a suitable sheath size that would directly affect a greater pulp yield. In terms of number of pods per bunch, it was observed a range of 3 to 9 pods per bunch. The pod diameter was very homogeneous (about 2 cm).

Fruit yield per tree varies markedly depending on tree age, genetic potential and edaphoclimatic conditions in the environment in which it develops (Feungchang et al., 1996). In Asia, young tamarind trees produce from 20 to 30 kg; while trees in full production (> 20 years) produce up to 200 kg (Chapman, 1984). The average yield per tree in India reported by Gunasena and Pushpakumara (2007) was 80 to 90 kg; with yield potential up to 263 kg when improved materials were used (Rao, 1995). In Mexico, yield varies from 150 to 222 kg; however trees with yields of up to 800 kg have been found (Orozco, 2001). Jambulingam and Fernández (1986) established that yield began to decline after the plant reached 50-year-old age.

Table 3
Descriptive statistical parameters of the quantitative traits of 32 individuals of tamarind selected in situ

Descriptor	Average	Varation coefficient (%)	Range	Minimum value	Maximum value
Tree age (year)	23.16	55.68	50	10	60
Plant height (m)	10.92	33.90	15	5	20
Trunk diameter (cm)	32.38	40.09	58	12	70
Number of seed/pod	3.97	24.36	4	2	6
Number of pod/bunch	5.81	22.47	6	3	9
Pod lenght (cm)	9.44	15.91	6	7	13
Pod diameter (cm)	2.28	20.02	1	2	3
Relation pulp/seed (%)	74.09	5.08	14	66	80
Pulp percentage	48.47	7.67	19	40	59
Fruit skin percentage	24.19	15.16	14	18	32
Seed percentage	25.72	10.60	12	20	32
Midrib percentage	1.63	69.46	5	0	5
Fruit yield (kg/tree)	66.56	47.39	118.90	22.92	141.82
Yield of pulp + seed (kg/tree)	53.18	47.05	95.45	18.19	113.64

According to the average data obtained in this study, the yield of the individuals analyzed was 66 kg; value that is much lower than those reported in the cited studies. These low yield could be influenced by the absence of agronomic management (fertilization, pruning, phytosanitary control, supplementary irrigation, use of floral inducers, among others) observed in the farmer field. It should be noted that 5 trees from the provinces of Manabí and one from Loja (TI-ECUM-002, TI-ECUM-007, TI-ECUM-009, TI-ECUM-026, TI-ECUL-32) reached yields superior to the 100 kg/tree, which could be considered promising.

Diallo et al. (2007) mentioned that greater genetic variation in a tamarind population would cause larger margin of action related to natural or artificial selection. On the other hand, Zetina et al. (2012) indicated that among the selection criteria of outstanding individuals for a program of domestication and genetic improvement were: healthy trees with a low level of alternation, showing large fruits, thick fruit skin, high pulp proportion, small seeds, trees in productive state (> 10 years of age in grafted trees and 13 years in seed trees) and a homogeneity of production in at least five years.

Grouping Structure

Hierarchical cluster analysis shows the relationship in degree of dissimilarity among the 32 selected individuals (Figure 2). Six groups were determined based on the characters evaluated, this result is similar to that found by Álvarez et al. (2018) in Colombia, who reported five groups; thus the morphological variability was similar. There was no clustering related to the geographical distribution of the accessions, thus groups were formed based on the characters evaluated as described below. Based on the

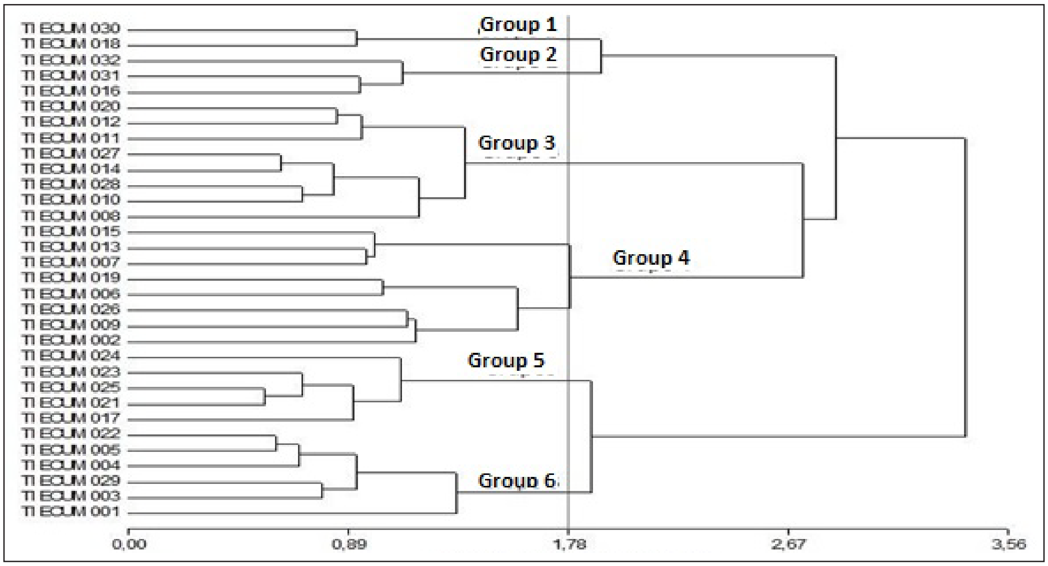

Figure 2. Dendrogram of the classification of 32 selected tamarind trees based on quantitative variables

genetic distance, it can be observed two major groups had little variation expressed at the agro-morphological level (phenotype). In addition, it is evident that groups 5 and 6 vary from the rest (groups 1 to 4).

The discriminant value of a quantitative character is the number of significant differences detected by the Duncan test, expressed as a fraction of the total number of possible comparisons within a group of accessions. This comparison allowed selecting five quantitative characters with greater discriminatory power. These variables were: plant height, this variable in group 2 was different from the rest except for group 1; number of seed per pod where group 1 had a significantly lower value than group 5; pulp percentage where groups 2 and 6 obtained the highest values; fruit skin percentage that showed group 3 different from the rest because of its superiority; and midrib percentage where group 1 was different from the rest because it showed the highest value (Table 4).

CONCLUSION

The abundance of *Tamarindus indica* in the Ecuadorian dry forest is scarce, mainly in individuals with outstanding phenotypic characteristics. The qualitative and quantitative traits suggest a relatively low genetic variability, related to the phenotypic heterogeneity of the selected trees. The most discriminant quantitative and qualitative variables that influenced the variability of the groups were plant height, number of seed/pod, pulp percentage, fruit skin percentage, midrib percentage, date of flowering and date of harvest. In order to identify individuals with outstanding

Table 4
Average values for quantitative characters based on the six groups formed

Descriptor	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Tree age (year)	11.00b	11.33b	28.25a	16.63ab	31.00a	28.50a
Plant height (m)[D]	8.00bc	6.50c	11.13b	10.75b	16.00a	9.83ab
Trunk diameter (cm)	30.50a	13.67b	35.38a	29.50a	40.80a	35.17a
Number of seed/pod[D]	3.00a	4.33bc	3.38ab	4.00abc	5.00c	4.00abc
Number of pod/bunch	4.50b	4.33b	6.25a	6.00ab	6.60a	5.50ab
Pod lenght (cm)	7.50b	10.00a	9.38ab	10.25a	9.80a	8,50ab
Pod diameter (cm)	2.00b	2.67a	2.50ab	2.38ab	2.00b	2.00b
Relation pulp/seed (%)	70.50b	78.33a	70.00b	75.13a	75.20a	76.33a
Pulp percentage[D]	42.50c	51.33a	45.88bc	49.38ab	49.20ab	50.67a
Fruit skin percentage[D]	24.00b	19.33a	28.38c	23.38b	23.60b	22.67ab
Seed percentage	28.50b	27.00ab	24.13a	26.00ab	26.00ab	25.67ab
Midrib percentage[D]	5.00c	2.67b	1.50a	1.25a	1.20a	1.00a
Fruit yield (kg/tree)	62.37b	93.90a	96.90a	59.06b	37.68b	47.91b
Yield of pulp + seed (kg/tree)	49.19b	76.16a	76.03a	47.85b	30.54b	38.53b

[D] Discriminant traits (show ranges abc) characteristics, an individual selection should be made in the areas where the accessions of groups 2 and 3 were located, considering characteristics such as lower tree height, initial age (about 11 years) of the productive stage and higher yields. This study could serve as a reference for breeding programs of this fruit tree; however, more basic scientific knowledge is needed to make this species attractive for farming and trading; as well as to introduce foreign germplasm to improve the genetic variability.

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REFERENCES

Álvarez, V., Muriel, S., & Costes, J. (2018). Morphoagronomic characterization of *Tamarindus indica* L. in orchards of tropical dry forest from Antioquia (Colombia). *Biodiversity International Journal*, *2*(5), 396-403.

Ballarin, M., & Raison, F. (2000). *Les reliques royales à Madagascar: source de légitimation et enjeu de pouvoir, XVIIIe-XXe siècles* [The royal relics in Madagascar: A source of legitimization and the issue of power, 18th-20th centuries]. Paris, France: Editions KARTHALA.

Caluwe, E., Halomova, K., & Van Damme, P. (2010). *Tamarindus indica* L. – A review of traditional uses, photochemistry and pharmacology. *Africa Focus*, *23*(1), 53-83.

Chapman, K. (1984). *Tamarind*. Brisbane, Australia: Department of Primary Industries.

Cruz, E., & Deras, H. (2000). Colecta de frutales tropicales en El Salvador [Collection of tropical fruit trees in El Salvador]. *Agronomía Mesoamericana*, *11*(2), 97-100.

Diallo, B., Joly, H., Mckey, D., Hossaert, M., & Chevallier, M. (2007). Genetic diversity of *Tamarindus indica* populations: Any clues on the origin from its current distribution?. *African Journal of Biotechnology*, *6*(7), 853-860.

Diallo, B., Mckey, D., Chevallier, M., Joly, H., & Hossaert, M. (2008). Breeding system and pollination biology of the semidomesticated fruit tree, *Tamarindus indica* L. (Leguminosae: Caesalpinioideae): Implications for fruit production, selective breeding, and conservation of genetic resources. *African Journal of Biotechnology*, *7*(22), 4068-4075.

El-Siddig, K., Gunasena, H., Prasad, B., Pushpakumara, D., Ramana, K., Vijayanand, P., & Williams, J. (2006). *Tamarind Tamarindus indica* L. Southampton, United Kingdom: Southampton Centre for Underutilised Crops.

Fandohan, B., Assogbadjo, A., Glele, R., Kyndt, T., & Sinsin, B. (2011). Quantitative morphological descriptors confirm traditionally classified morphotypes of *Tamarindus indica* L. *Genetic Resources and Crop Evolution*, *58*(2), 299–309. doi: 10.1007/s10722-010-9575-3

Fandohan, B., Assogbadjo, A., Glele, R., Sinsin, B., & Van Dame, P. (2010). Impact of habitat type on the conservation status of tamarind (*Tamarindus indica* L.) populations in the W National Park of Benin. *Fruits*, *65*(1), 11–19. doi: 10.1051/fruits/2009037

Feungchang, S., Yimsawat, T., Chindaprasert, S., & Kitpowsong, P. (1996). Tamarind (*Tamarindus indica* L.) plant genetic resources in Thailand. *Thai Journal of Agricultural Science*, *1*(1), 1-11.

Graf, B., Rojas, P., & Baldeón, M. (2016). Discovering the pharmacological potential of Ecuadorian market plants using a screens-to-nature participatory approach. *Journal of Biodiversity, Bioprospecting and Development*, *3*(1), 1-9. doi: 10.4172/2376-0214.1000156

Gunasena, H., & Pushpakumara, D. (2007). *Tamarind* – Tamarindus indica. Nueva Delhi, India: World Agroforestry Centre.

International Plant Genetic Resources Institute. (1980). *Tropical fruit descriptor*. Bangkok, Thailand: Author.

Jambulingam, R., & Fernández, C. (1986). Multipurpose trees and shrubs on farmlands in Tamil Nadu State (India). *Agrofosrestry Systems*, *4*(1), 17-32. doi: 10.1007/BF01834699

Orozco, S. (2001). *El cultivo del tamarindo* (Tamarindus indica *L.*) *en el trópico seco de México* [The cultivation of tamarind (*Tamarindus indica* L.) in the dry forest of Mexico]. Tecomán, México: SAGARPA.

Rao, S. (1995). Tamarind economics. *Spice in India*, *8*(4), 1-11.

Sarmiento, L., Pérez, I., Díaz, B., Álvarez, H., & Viera W. (2017). Molecular marker-based characterization of Ecuadorian dry forest tamarind plus trees. *Bioagro*, *29*(3), 153-162.

Tapia, F., Mercador, P., & Gómez, S. (2012). Contribución a la citogenética de *Tamarindus indica* (Leguminosae: Caesalpinioideae) [Contribution to the cytogenetics of *Tamarindus indica* (Leguminosae: Caesalpinioideae)]. *Acta Botánica Mexicana*, *98*(1), 99-110.

Troup, R. (1921). *The silviculture of Indian trees*. Gloucester, United Kingdom: Clarendon Press.

Vásquez, V., Pérez, M., Osuna, J., & Urías, M. (2009). Intensidad de poda sobre el vigor, producción y peso del fruto, del mango 'Ataulfo' [Intensity of pruning on the vigor, production and weight of the fruit, of the mango 'Ataulfo']. *Revista Chapingo*, *15*(2), 127-132.

Zetina, R., Del Angel, A., Becerra, E., & Rebolledo, A. (2012). *Tecnología para la producción sustentable de tamarindo (Tamarindus indica L.) en el estado de Veracruz* [Technology for the sustainable production of tamarind (*Tamarindus indica* L.) in the State of Veracruz]. Veracruz, México: Alfa y Omega.

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Liquid Organic Fertilizer from Plant Extracts Improves the Growth, Yield and Quality of Sweet Corn (*Zea mays* L. var. *saccharata*)

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ABSTRACT

The objective of this research was to study the effect of concentration and dose of liquid organic fertilizer (LOF) derived from an extract mixture derived from *lamtoro* leaves, banana humps, and coconut fibers on the growth, yield, quality, and nutrient uptake of sweet corn. Experiment 1 comprised six treatments, namely a control without LOF, recommended inorganic fertilizers, and LOF at a concentration of 15 ml l^{-1}, 30 ml l^{-1}, 45 ml l^{-1}, or 60 ml l^{-1}. Experiment 2 consisted of five treatments, namely recommended inorganic fertilizers, and LOF doses of 25 l ha^{-1}, 50 l ha^{-1}, 75 l ha^{-1} or 100 l ha^{-1}. Results showed that LOF consistently increased the growth, yield and quality of sweet corn. Application of LOF with a concentration of 60 ml l^{-1} or a dose of 100 l ha^{-1} showed the highest yield compared to other treatments. The quality of sweet corn increased markedly. It was concluded that LOF could be applied as an additional supplement to inorganic fertilizers used for sweet corn organic farming in the tropics.

Keywords: Banana hump, coconut fiber, leucaena extract, nutrient uptake, organic farming

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INTRODUCTION

The community prefers sweet corn (*Zea mays* L. var. *saccharata*) because of its relatively high sugar content. Organic sweet corn production needs can be improved by improving fertilization techniques. Fertilizers for sweet corn can be given in the solid or liquid form. Solid organic fertilizers tend to be taken up by the crop

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relatively slowly (Hartz et al., 2000; Johnson et al., 2012). Another alternative in organic tropical agriculture is the use of liquid organic fertilizers (LOF), which are taken up more quickly by crops.

The application of fertilizer into the plants through leaves was successfully carried out on corn plants (Amanullah et al., 2014). Similarly, the application of LOF was carried out on various vegetable crops such as sweet corn (Fahrurrozi et al., 2016) and tomatoes (Zhai et al., 2009). Hartz et al. (2010) reported that the application of LOF could function the same as conventional fertilization with solid fertilizers. Furthermore, the researchers stated that nitrogen derived from liquid organic fertilizers was more readily available and the nitrification process took place faster.

One cultivation technique used to increase growth, yield, and quality of sweet corn grown organically are LOF made from the waste of plant materials available around the farmer's garden. There is still a lack of a scientific basis on the utilization of plant extracts as LOF. Plant wastes such as *lamtoro* (*Leucaena leucocephala*) leaves, banana humps, and coconut husks are widely available around farmers' gardens in tropical areas. *Lamtoro* leaves are often used as animal feed. *Lamtoro* leaves contain significant amounts of nitrogen, protein, vitamins, and minerals (Farinu et al., 1992; Meulen et al., 1979). Liquid organic fertilizer from the leaves of *lamtoro* is a source of nitrogenous nutrients for plants. Banana humps contain microbial decomposers of organic matter, such as *Bacillus* sp., *Aeromonas* sp. and *Aspergillus niger* (Suhastyo et al., 2013). These microbes are able to decompose organic matter. The main content of coconut fibers is cellulose, hemi-cellulose, and lignin (Arsyad et al., 2015). If the coconut fiber is soaked and fermented with water, the potassium contained in coconut fiber will dissolve in soaking water so that the liquid fertilizer coconut husk is high in potassium.

The purpose of this study was to determine the effect of concentration and dose of liquid organic fertilizer made from an extract mixture of *lamtoro* leaves, banana humps and coconut fibers on the growth, production, quality, and nutrient uptake of sweet corn plants.

MATERIALS AND METHODS

This research was carried out in Kota Sepang experimental station, Bandar Lampung, Indonesia. The study area was classified as having Ultisol soil. A field study was set up in RCBD (Randomized Complete Block Design) and replicated three times. The experiment consisted of two sub-experiments. Experiment 1 (April to June 2016) consisted of six treatments, namely an unfertilized control, recommended inorganic fertilizer, and application of LOF with a concentration of 15 ml l^{-1}, 30 ml l^{-1}, 45 ml l^{-1}, or 60 ml l^{-1}. Experiment 2 (April to June 2017) consisted of five treatments, namely recommended inorganic fertilizer, and an application of LOF with a dose of 25 l ha^{-1}, 50 l ha^{-1}, 75 l ha^{-1}, or 100 l ha^{-1}.

The research began with making plant extract LOF following a modified procedure developed by Astuti et al. (2014). *Lamtoro* leaves, banana humps, and coconut fibers were cut into small pieces and then separately put into containers, into which brown sugar and rice washing water were added and stirred evenly. Then EM-4 was added, after which the container was closed and then fermented for 21 days and filtered. Liquid organic fertilizer from *lamtoro* leaves, banana humps, and coconut fibers was then mixed in a ratio of 1: 1: 1. The nutrient content of the plant extracts (Table 1) was analyzed in Soil Laboratory, Faculty of Agriculture, University of Lampung.

Table 1
Content of fermented plant extract

Plant extract	N (ppm)	P (ppm)	K (ppm)
Lamtoro leaves	763.01	55.11	125.81
Banana humps	238.04	63.88	88.21
Coconut fiber	133.12	8.95	192.11

All plots were hand hoed before planting with sweet corn. The area of each plot was 3 m × 3 m, and the row spacing was 70 cm × 20 cm. The application of liquid fertilizer was carried out by spraying evenly on the upper and lower part of leaves. The application of LOF was carried out once a week from 2 to 7 weeks after planting (WAP). The inorganic recommended fertilizers were given included urea at 300 kg ha^{-1}, SP-36 at 150 kg ha^{-1}, and KCl at 100 kg ha^{-1}. Agronomic practices used in this study included watering, growing, control of weeds, pests, and diseases organically. Harvesting was conducted at 70 days after planting.

The parameters observed in this study were the number of leaves, stem diameter, plant height, leaf area index (LAI), SPAD value, the weight of husked ear, the weight of unhusked ear, ear length, ear diameter, shoot dry weight, ºBrix value, yield, and N, P and K nutrient uptake. Statistical analysis of the data was conducted using MINITAB v. 16.0 software. Analysis of variance and least significant difference (LSD) tests was used to test overall treatment effects and between-treatment differences and was conducted at the 5% probability level.

RESULTS AND DISCUSSION

Results

The LOF application affected the vegetative parameters of the plants. Leaf number and stem diameter were greater in LOF-applied plant than the control (Table 2). The results of the analysis of variance showed that the application of LOF increased the number of leaves and stem diameter. The number of leaves and stem diameter treated with concentrations of 15 ml l^{-1}, 30 ml l^{-1}, 45

Table 2
Effects of liquid organic fertilizer concentration on the number of leaves and stem diameter in experiment 1

LOF concentration	Number of leaves	Stem diameter (cm)
Control	11.33 c	1.60 c
Inorganic fertilizer	12.47 b	1.80 b
15 ml l^{-1}	12.80 ab	1.93 ab
30 ml l^{-1}	13.00 ab	1.89 ab
45 ml l^{-1}	13.20 a	2.02 a
60 ml l^{-1}	13.47 a	2.00 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

ml l^{-1}, and 60 ml l^{-1} were not significantly different. The lowest number of leaves and the lowest stem diameter were recorded in the control treatment (Table 2).

The LOF application increased plant height, leaf area index, and the greenness of the leaves (indicated by SPAD value) (Table 3). The plant height, LAI, and SPAD value were greater in LOF-applied plants than with the inorganic fertilizer treatment. Plant height and LAI increased with increasing doses of LOF until 75 l ha^{-1}; above these levels, there was no significant additional increase. The greenness level of leaves (SPAD value) in the 100 l ha^{-1} treatment was higher than in the other treatments, while doses of 50 l ha^{-1} and 75 l ha^{-1} were not significantly different from each other. The lowest plant height, LAI and SPAD value were recorded with the inorganic fertilizer treatment (Table 3).

LOF application also affected the generative parameters of plants. The results of the analysis of variance indicated that the LOF application increased the weight of unhusked ears and weight of husked ears (Tables 4 and 5). Weights of unhusked ears and weight of husked ears in the LOF treatment were significantly higher than the control treatment (Table 4). The lowest weights of unhusked ears, the weight of husked ears, ear length, and ear diameter were recorded in the control plants (Table 4).

The LOF application increased ear length (Tables 4 and 5). Ear lengths at concentrations of 30 ml l^{-1}, 45 ml l^{-1}, and 60 ml l^{-1} were not significantly different from the treatment of inorganic fertilizer (Table 4), while ear length between doses of 25 l ha^{-1} and inorganic fertilizer did not show any significant differences (Table 5). The ear length between doses of 50 l ha^{-1}, 75 l ha^{-1}, and 100 l ha^{-1} did not show any significant differences (Table 5).

Table 3
Effects of liquid organic fertilizers doses on plant height, LAI, and SPAD value in experiment 2

LOF dose	Plant height (cm)	LAI	SPAD value
Inorganic fertilizer	82.05 c	3.07 d	44.28 c
25 l ha^{-1}	83.31 b	3.64 c	44.91 c
50 l ha^{-1}	84.56 b	4.23 b	46.22 b
75 l ha^{-1}	93.80 a	5.33 a	46.97 b
100 l ha^{-1}	93.45 a	5.51 a	48.15 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

Table 4
Effects of liquid organic fertilizer concentration on the weight of husked ear, the weight of unhusked ear, ear length, and ear diameter in experiment 1

LOF concentration	Weight of unhusked ear (kg $plant^{-1}$)	Weight of husked ear (kg $plant^{-1}$)	Ear length (cm)	Ear diameter (cm)
Control	1.88 b	1.28 b	18.61 c	3.94 c
Inorganic fertilizer	2.33 a	1.77 a	21.03 ab	4.42 b
15 ml l^{-1}	2.35 a	1.78 a	20.15 b	4.40 b
30 ml l^{-1}	2.30 a	1.97 a	21.14 ab	4.55 ab
45 ml l^{-1}	2.63 a	2.08 a	21.85 a	4.71 a
60 ml l^{-1}	2.63 a	2.12 a	21.61 a	4.68 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

Table 5
Effects of liquid organic fertilizer doses on the weight of husked ear, the weight of unhusked ear, ear length, and ear diameter in experiment 2

LOF dose	Weight of unhusked ears (kg plant^{-1})	Weight of husked ears (kg plant^{-1})	Ear length(cm)	Ear diameter (cm)
Inorganic fertilizer	2.10 e	1.60 c	20.32 a	4.39 d
25 l ha^{-1}	2.30 d	1.73 c	20.19 a	4.43 c
50 l ha^{-1}	2.57 c	1.80 bc	18.45 b	4.50 b
75 l ha^{-1}	3.03 b	2.07 ab	18.12 b	4.58 a
100 l ha^{-1}	3.30 a	2.27 a	17.79 b	4.60 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

The application of LOF increased the ear diameter (Tables 4 and 5). The lowest ear diameter was recorded in control plants (Table 4). The diameter of the ear between concentrations of 30 ml l^{-1}, 45 ml l^{-1}, 60 ml l^{-1} (Table 4) as well as between doses of 75 l ha^{-1} and 100 l ha^{-1} (Table 5) were not significantly different.

The application of LOF consistently increased sweet corn yields (Tables 6 and 7). The yield of sweet corn at all LOF concentrations (Table 6) was not significantly different. The lowest yield was recorded in control treatment. The highest yield was obtained at a concentration of 60 ml l^{-1} (Table 6) and dosed 100 l ha^{-1} (Table

Table 6
Effects of liquid organic fertilizers concentration on shoot dry weight, ºBricks value, and yield in experiment 1

LOF concentration	Shoot dry weight (g plant^{-1})	ºBrix value	Yield (ton ha^{-1})
Control	14.19 a	15.22 c	8.28 b
Inorganic fertilizers	19.24 bc	15.76 b	11.59 a
15 ml l^{-1}	17.16 ab	15.85 ab	13.26 a
30 ml l^{-1}	18.97 bc	16.16 ab	13.24 a
45 ml l^{-1}	20.74 c	16.18 a	14.76 a
60 ml l^{-1}	21.31 c	16.22 a	14.94 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

Table 7
Effects of dose of liquid organic fertilizers doses on shoot dry weight, ºBricks value, and yield in experiment 2

LOF dose	Shoot dry weight (g plant^{-1})	ºBrix value	Yield (ton ha^{-1})
Inorganic fertilizers	16.25 d	11.05 b	15.15 c
25 l ha^{-1}	16.87 c	11.50 b	15.97 b
50 l ha^{-1}	17.07 b	11.56 b	16.21 b
75 l ha^{-1}	17.43 a	12.39 a	17.37 a
100 l ha^{-1}	17.33 a	12.56 a	17.81 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

7). The application of LOF increased the dry shoot weight (Tables 6 and 7). The lowest shoot dry weight was noted in control treatment (Table 6).

Application of LOF increased plant nutrient uptake (Table 8). Nutrient uptake of P and K in the LOF treatment were significantly higher than those treated with inorganic fertilizer. Also, nutrient uptake of N, P, and K between doses of 25 l ha^{-1} and 50 l ha^{-1} as well as between doses 75 l ha^{-1} and 100 l ha^{-1} did not show any significant differences (Table 8).

The LOF application increased the quality of sweet corn as indicated by sugar content (Tables 6 and 7) as indicated by ºBrix value. The lowest sugar content was recorded in control treatment (Table 6). The sugar content between all concentrations of LOF (Table 6) and between doses of 75 l ha^{-1} and 100 l ha^{-1} (Table 7) were not significantly different.

Table 8
Effects of liquid organic fertilizers doses on nutrient uptake in experiment 2

LOF concentration	N uptake (g plant^{-1})	P uptake (g plant^{-1})	K uptake (g plant^{-1})
Inorganic fertilizers	7.70 b	1.97 c	2.51 c
25 l ha^{-1}	8.17 b	2.39 b	2.69 b
50 l ha^{-1}	8.57 b	2.51 b	2.93 ab
75 l ha^{-1}	9.80 ab	2.70 a	3.22 a
100 l ha^{-1}	10.10 a	2.72 a	3.47 a

In a column, values having common letter(s) do not differ significantly at $p \leq 0.05$ as per LSD

Discussion

Vegetative growth of plants is represented by the number of the parameters of leaves, stem diameter, and plant height. The highest number of leaves and stem diameter were seen concentrations of 45 ml^{-1} and 60 ml l^{-1} in experiment 1. At the beginning of growth, plants can absorb nutrients from LOF, which are used by plants to support growth. Nitrogen (Table 1) is needed for vegetative growth. Nitrogen is a key element for producing vegetative biomass on the early growth of maize (Massignam et al., 2009).

The fact that LOF increased the leaf area index might be due to an increase in the number of leaves (Table 3). Fageria et al. (2009) stated that foliar application required higher LAI for absorbing applied nutrient solution in sufficient amounts. Table 3 shows that the application of LOF at a dose of 100 l ha^{-1} gave a higher value of leaf greenness compared to the other treatments. According to Nugroho (2015) nitrogen, in addition to stimulating plant growth, also gives the green color of the leaves. The darker the green color of leaves on corn plants, the higher the amount of nitrogen absorbed by the plants.

The application of LOF gave positive results in terms of the generative growth of plants. The results showed that the concentration of 60 ml l^{-1} gave the best results in terms of the weight of unhusked ears and husked ears, and the treatment with a dose of 100 l ha^{-1} gave the highest ear diameter and the heaviest weight of the ears. It can be inferred that the higher the dose or concentration of LOF given, the higher the generative growth of plants. According to Zafar et al. (2013), enlargement of ear diameter is related to the availability of phosphorus; phosphorus greatly influences the formation of ears.

Liquid organic fertilizer application increased the yield of sweet corn. This means that the contribution of nutrients from LOF can be complementary to the recommended solid fertilizer. The results of this study are supported in various other LOF studies. Minardi et al. (2015) found that banana corm extract increased the available P in soil. Aini et al. (2017) stated that the treatment of banana humps had a significant effect on the growth and yield of soybean. Working with *Gleicheni linearis* plant extract, Aulya et al. (2018) showed that the application of 100 mg l^{-1} was the most effective concentration in increasing plant height and leaf area of maize compared to a control.

The results showed that the treatment of a dose of LOF 100 l ha^{-1} gave a high degree of N, P, and K nutrient uptake, and this was attributed to the adequate supply of nutrients from LOF. This means that the higher the dose of LOF application, the higher the nutrient uptake of N, P, and K; in contrast to Muktamar et al. (2016) showed that increase in rates of LOF significantly raised nitrogen uptake by sweet corn but did not raise phosphorus and potassium uptake. Mukuralinda et al. (2010) also showed high P nutrient uptake due to the application of organic fertilizer compared to control.

The application of N nutrients in plants has a direct role in the formation of amino acids, proteins, nucleic acids, enzymes, nucleoproteins, and alkaloids (Mokhele et al., 2012), which are needed for the vegetative growth process of plants and increase the greenness of leaves. Leaf N nutrient uptake has a close relationship with the leaf greenness level, characterized by the SPAD value. Increasing nutrient uptake of P enhanced the metabolic processes in the plant to become more active. P is known as the forming element of ATP for energy sources. In addition to P, K also plays an important role in improving the quality of plant fruit. The application of liquid organic fertilizer containing an element of K from coconut fiber extract improved the quality of sweet corn, which is characterized by a higher Brix value. Jifon and Lester (2009) found that the application of K fertilizer through leaves increased the sugar content of muskmelon plants. Sweet taste in sweet corn involves potassium nutrients, which play a role in the activation of many enzymes that has a role in metabolic processes in plants. Enzymes that play a role in sugar synthesis are activated by K (Prajapati & Modi, 2012).

The research showed that in both experiments, between the all concentration of the LOF and inorganic recommended fertilizers (experiment 1) as well as between the doses of the LOF and inorganic recommended fertilizers (experiment 2) gave the same effect on all parameters of growth and yield of sweet corn. Sweet corn plants fertilized with LOF had the same growth and yield with those of recommended fertilizers. Therefore, it implied that LOF could be a complementary fertilizer. Many researchers have also succeeded in applying the foliar application of LOF to sweet corn (Muktamar et al., 2017) and corn (Aulya et al., 2018).

From this experiment, it can be recommended to utilize local plant resources around farmers such as *lamtoro* leaves, banana humps, and coconut fibers to be used as LOF. The high content of N, P, K nutrients in LOF (Table 1) means that the application of liquid organic fertilizer is sufficient to meet the needs of N, P, and K nutrients for sweet corn plants. The mixture of plant residues contained the basic nutrient requirements for plants. *Lamtoro* leaves have a high content of protein, carbohydrate, and nitrogen (Devi et al., 2013). Banana humps contained P and K (Bahtiar et al., 2016). *Azospirillum*, *Azotobacter*, *Bacillus* sp., *Aeromonas* sp., and *Aspergillus niger* and the other phosphate solubilizing bacteria were identified in banana humps (Minardi, et al., 2015; Suhastyo et al., 2013). Banana humps naturally had bioactive compounds to be used as plant growth regulators (Ulfa et al., 2013), namely auxin, gibberellin and cytokinin. Coconut fiber had a high P and K (Abad et al., 2002). It can be said that the application of LOF supplied nutrients, microbes, and hormones leading to better growth, yield and quality of sweet corn.

The use of liquid organic fertilizer from plant materials can be used as a complementary fertilization technology in developing organic agriculture. However, the nutrient content of plant extract is dependent on the type of organic waste used, the fermentation period, and the storage of plant extract. In its application, liquid organic fertilizer needs to be mixed with a surfactant to increase the efficiency of uptake by plant leaves. The application of liquid organic fertilizer is an inexpensive and effective technology in organic farming. Therefore, local-based liquid organic fertilizers that contain extracts of *lamtoro*, banana hump, and coconut fiber have the potential to support organic farming. Further research is still needed to determine the appropriate concentration and doses of liquid organic fertilizer for each horticultural and food crop.

CONCLUSIONS

Application of LOF with the concentration of 60 ml l^{-1} or dose 100 l ha^{-1} showed the best growth, yield, and quality of sweet corn compared to lower concentrations or lower doses. Application of LOF improved the agronomic quality of sweet corn. Sweet corn sprayed with LOF absorbed a higher amount of N, P, and K. It is recommended that LOF could be a complementary fertilization technology in sweet corn organic farming.

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REFERENCES

Abad, M., Noguera, P., Puchades, R., Maquieira, A., & Noguera, V. (2002). Physico-chemical and chemical properties of some coconut coir dusts for use as a peat substitute for containerised ornamental plants. *Bioresource Technology*, *82*(3), 241-245. doi: 10.1016/S0960-8524(01)00189-4

Aini, D. N., Sugiyanto, B., & Herlinawati. (2017). Application of local microorganism derived from stem of banana and goat manure on the yield of soybean (*Glycine max* L. Merrill) var. Baluran. *Agriprima, Journal of Applied Agricultural Sciences*, *1*(1), 35-43. (In Indonesian) doi: 10.25047/agriprima.v1i1.13

Amanullah, Kakar, K. M., Khan, A., Khan, I., Shah, Z., & Hussain, Z. (2014). Growth and yield response of maize (*Zea mays* L.) to foliar NPK-fertilizers under moisture stress condition. *Soil Environment*, *33*(2), 116-123.

Arsyad, M., Wardana, I. N. G., Pratikto, & Irawan, Y. S. (2015). The morphology of coconut fiber surface under chemical treatment. *Revista Materia*, *20*(1), 169-177.

Astuti, T., Amir, Y. S., Yelni, G., & Isyaturriyadhah (2014). The result of biotechnology by local microorganisms to banana peel on rumen fluid characteristics as ruminant feed. *Journal of Advanced Agricultural Technologies*, *1*(1), 28-31. doi:10.12720/joaat.1.1.28-31

Aulya, N. R., Noli, Z. A., Bakhtiar, A., & Mansyurdin. (2018). Effect of plant extracts on growth and yield of maize (*Zea mays* L.). *Pertanika Journal of Tropical Agricultural Sciences*, *41*(3), 1193-1205.

Bahtiar, S. A., Muayyad, A., Ulfaningtias, L., Anggara, J., Priscilla, C., & Miswar. (2016). Compost use banana weevil (*Musa acuminata*) to boost growth and content of sugar sweet corn (*Zea mays* L. *saccharata*). *Agritrop, Journal of Agricultural Science*, *14*(1), 18-22. (In Indonesian)

Devi, M. V., Ariharan, V., & Prasad, P. N. (2013). Nutritive value and potential uses of *Leucaena leucocephala* as biofuel - A mini review. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *4*(1), 515-521.

Fageria, N. K., Filho, M. P. B., Moreira, A., & Guimarães, C. M. (2009). Foliar fertilization of crop plants. *Journal of Plant Nutrition*, *32*(6), 1044–1064. doi:10.1080/01904160902872826

Fahrurrozi, Muktamar, Z., Dwatmadji, Setyowati, N., Sudjatmiko, S., & Chozin, M. (2016). Growth and yield responses of three sweet corn (*Zea mays* L. var. *saccharata*) varieties to local-based liquid organic fertilizer. *International Journal on Advanced Science Engineering and Information Technology*, *6*(3), 319-323.

Farinu, G. O., Ajiboye, S. O., & Ajao, S. (1992). Chemical composition and nutritive value of leaf protein concentrate from *Leucaena leucocephala*. *Journal of the Science of Food and Agriculture*, *59*(1), 127-129. doi: 10.1002/jsfa.2740590119

Hartz, T. K., Mitchell, J. P., & Giannini, C. (2000). Nitrogen and carbon mineralization dynamics of manures and composts. *HortScience*, *35*(2), 209–212. doi: 10.21273/HORTSCI.35.2.209

Hartz, T. K., Smith, R., & Gaskell, M. (2010). Nitrogen availability from liquid organic fertilizers. *HortTechnology*, *20*(1), 169-172. doi: 10.21273/HORTTECH.20.1.169

Jifon, J. L., & Lester, G. E. (2009). Foliar potassium fertilization improves fruit quality of field-grown muskmelon on calcareous soils in south Texas. *Journal of the Science of Food and Agriculture*, *89*(14), 2452–2460. doi: 10.1002/jsfa.3745

Johnson, H. J., Colquhoun, J. B., Bussan, A. J., & Laboski, C. A. M. (2012). Estimating nitrogen mineralization of composted poultry manure, organic fertilizers, and green manure crops for organic sweet corn production on a sandy soil under laboratory conditions. *HortTechnology*, *22*(1), 37-43. doi: 10.21273/HORTTECH.22.1.37

Massignam, A. M., Chapman, S. C., Hammer, G. L., & Fukai, S. (2009). Physiological determinants of maize and sunflower grain yield as affected by nitrogen supply. *Field Crops Research*, *113*(3), 256-267. doi: 10.1016/j.fcr.2009.06.001

Meulen, U., Struck, S., Schulke, E., & Harith, E. A. E. (1979). A review on the nutritive value and toxic aspects of *Leucaena leucocephala*. *Tropical Animal Production*, *4*(2), 113-126.

Minardi, S., Hartati, S., Widijanto, H., & Wulandari, D. (2015). The use of extract banana corm and phosphate rock to increase available-P in Alfisols. *Sains Tanah, Journal of Soil Science and Agroclimatology*, *12*(2), 76-81. doi: 10.15608/stjssa

Mokhele, B., Zhan, X., Yang, G., & Zhang, X. (2012). Nitrogen assimilation in crop plants and its affecting factors. *Canadian Journal of Plant Science*, *92*(3), 399-405. doi: 10.4141/CJPS2011-135

Muktamar, Z., Fahrurrozi, Dwatmadji, Setyowati, N., Sudjatmiko, S., & Chozin, M. (2016). Selected macronutrients uptake by sweet corn under different rates liquid organic fertilizer in closed agriculture system. *International Journal on Advanced Science Engineering and Information Technology*, *6*(2), 258-261.

Muktamar, Z., Sudjatmiko, S., Chozin, M., Setyowati, N., & Fahrurrozi. (2017). Sweet corn performance and its major nutrient uptake following application of vermicompost supplemented with liquid organic fertilizer. *International Journal on Advanced Science Engineering and Information Technology*, *7*(2), 602- 608.

Mukuralinda, A., Tenywa, J. S., Verchot, L., Obua, J., Nabahungu, N. L., & Chianu, J. N. (2010). Phosphorus uptake and maize response to organic and inorganic fertilizer inputs in Rubona, Southern Province of Rwanda. *Agroforestry Systems*, *80*(2), 211-221. doi:10.1007/s10457-010-9324-9

Nugroho, W. S. (2015). Determination of a standard of green color on corn (*Zea mays* L.) leaves as the marker of nitrogen adequacy on Regosol soil. *Planta Tropika, Journal of Agro Science*, *3*(1), 8-15. (In Indonesian) doi: 10.18196/pt.2015.034.8-15

Prajapati, K., & Modi, H. A. (2012). The importance of potassium in plant growth - A review. *Indian Journal of Plant Sciences*, *1*(2), 177-186.

Suhastyo, A. A., Anas, I., Santosa, D. A., & Lestari, Y. (2013). Studies of microbiology and chemical properties of the local microorganisms (MOL) used in rice farming by SRI method (System of Rice Intensification). *Sainteks*, *10*(2), 29-39. (In Indonesian)

Ulfa, F., Sengin, E. L., Baharuddin, Syaiful, S. A., Sennang, N. R., Rafiuddin, Nurfaida, Ifayanti. (2013). Potential of plant extracts as growth exogenous regulators of potato seeds. *International Journal of Agriculture Systems*, *1*(2), 98-103.

Zafar, M., Abbasi, M. K., & Khaliq, A. (2013). Effect of different phosphorus sources on the growth, yield, energy content and phosphorus utilization efficiency in maize at Rawalakot Azad Jammu and Kashmir Pakistan. *Journal of Plant Nutrition*, *36*(12), 1915-1934. doi:10.1080/01904167.2013.819892

Zhai, Z., Ehret, D. L., Forge, T., Helmer, T., Lin, W., Dorais, M., & Papadopoulos, A. P. (2009). Organic fertilizers for greenhouse tomatoes: Productivity and substrate microbiology. *HortScience*, *44*(3), 800-809. doi: 10.21273/HORTSCI.44.3.800

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Short communication

Oviposition Behavior of *Scirpophaga incertulas*, the Yellow Stemborer (Lepidoptera: Crambidae) in A Non-Choice Study

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ABSTRACT

Scirpophaga incertulas, or the yellow stemborer, is a dominant paddy pest in Asia and contributes to great yield loss in paddy cultivation. Breeding paddy varieties that are resistant to yellow stemborer is an eco-friendly alternative for managing this pest rather than using hazardous chemical insecticides. In this non-choice study, the oviposition behaviour of female yellow stemborers on three different local Sarawak paddy landraces was observed. The number and size of egg masses found on the three local paddy landraces, namely Bajong, Bubok, and Bario, were similar. In general, the yellow stemborers of this study preferred to oviposit on the leaves instead of stems. On leaves, the number of egg masses oviposited on the abaxial side and adaxial side of leaves was comparable. Based on this preliminary data, the three local paddy landraces may not be good candidates in a paddy breeding program that resists towards yellow stemborers.

Keywords: Antixenosis resistance, egg mass position, paddy plant, yellow stemborer

INTRODUCTION

Rice stemborers are a group of dominant paddy pests. In Sarawak, a statewide rice pests survey carried out from 2009 to 2011 in 166 rice fields showed that 11.4 % of rice damage in the fields was caused by rice stemborers (Gumbek & Hamsein, 2011). The damages are caused by the larvae of rice stemborers that bore into paddy tillers and feed on the inner cells. Eventually, the infestation will cause whitehead and

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deadhearts, *i.e.*, grain reduction per plant/hill. One of the rice stemborer species causing yield reduction is the yellow stemborer (*Scirpophaga incertulas* Walker).

Paddy landraces that resist to yellow stemborers are valuable genetic resources. They can be good candidates for a resistance breeding program. Generally, a plant variety is said to be resistant toward certain insect pests when they have heritable traits that contribute to the avoidance of pests from the plant (antixenosis), a reduction in the survival rate of the pest (antibiosis) or tolerance to the infestation of the pest (Smith & Clement, 2012). Sarawak is blessed with diverse paddy landraces that not only vary in the colours of their husks and grains and their aroma, but they are also known to be more resistance to various biotic and abiotic stresses (Yeo et al., 2018). These are precious genetic resources for rice breeding in Sarawak. Their resistance towards yellow stemborer is understudied, not to mention the oviposition behaviour of yellow stemborer on these paddy landraces. In order to study the resistance of Sarawak paddy landraces against yellow stemborer, the oviposition behaviour of yellow stemborer on three Sarawak paddy landraces was investigated in a non-choice test.

METHODS

Three local paddy landraces were selected for this study, *viz.* Bajong (Husk: dark brown; Kernel: black), Bario (Husk: yellow; Kernel: white), and Bubok (Husk: yellow; Kernel: white). These paddy landraces are among the famous landraces planted in Sarawak. They are socially and economically important to the local farmers. Local farmers plant these paddy landraces as their main food source (Teo, 2010). Among the three paddy landraces, Bario rice has high export potential with high nutritive values (Nicholas et al., 2014).

The paddy seeds were provided by the Agriculture Research Centre, Semongok, Sarawak. They were soaked in water to promote germination. Approximately 50 germinated seeds were transferred into trays (30 cm x 20 cm) containing planting medium (2 topsoil: 1 sand). One-month-old seedlings (three-leaf stage) were transplanted into pots (3 gallons) with five seedlings per pot. Nitrogen-phosphorus-potassium compound growth-promoting fertilizer was applied to each of the paddy plant pots once in every two weeks following the manufacturer's instruction. Two-month-old paddy plants were later used for the experiment. Three paddy landraces, each with 25 replicates, were planted, totaling to 75 pots.

The paddy field in Kampung Skuduk-Chupak (1°15'07.5"N, 110°26'17.2"E) was chosen as the stemborer sampling site. The 75 pots of paddy plants were brought to the field in a random order. Each pot was covered with a high-density polyethylene (HDPE) plastic mesh (diameter = 3 mm). A white screen light trap was set up next to the paddy field from 7 pm to 10 pm. Adult females of yellow stemborer were picked from the white screen. Ten were released into each pot of paddy plants through a small opening on the HDPE plastic mesh. Infested pots were brought back from the paddy field and arranged randomly in the designated open field area for plant experimental studies on campus. Yellow stemborer sampling was completed within one month in order to infest all 75 pots of paddy plants with yellow stemborers.

The number of egg masses laid (oviposition) for each pot was counted at the fifth day of the experiment. A one-way ANOVA test (α=0.05) was conducted by using SPSS software to test the difference between the number of egg masses deposited on the plants of three paddy landraces. The positions of the egg masses were recorded and categorized into egg masses laid on either plant surfaces or on non-plant surfaces. Eggs laid on plant surfaces were further divided into stems and leaves (abaxial or adaxial). The egg masses found on the walls of the pot or the plastic mesh were counted as egg mass laid on non-plant surface. The significant difference between the mean of egg masses deposited on plant surface versus non-plant surface, leaves versus stems, and abaxial versus adaxial side on the leaves, were determined by using a two-sample t-test (α=0.05) in SPSS software, regardless of the paddy landraces. In the analysis, only 73 pots were considered while two pots from the Bubok landrace were unsuccessful in infestation.

RESULTS AND DISCUSSION

A total of 810 egg masses were counted on plant surfaces and another 77 egg masses were found on the non-plant surfaces. Most of the egg masses were similar in size, with a diameter between 3 mm and 6 mm. All the egg masses found on the plant were intact, and the abnormalities described by Hilker and Meiners (2006) were not observed on the egg masses (mortality) as well as on the contact sites of the egg masses and leaf surface (necrosis and neoplasm). This may indicate that there was no egg-induced response or antibiosis resistance among the three paddy landraces. Further investigation can be done to study the hatching rate of the egg masses, survival rate and growth of larvae to understand more about the antibiosis resistance of these local paddy landraces.

There was no significant difference in the total number of egg masses laid on the three paddy landraces (One-way ANOVA; $F_{2,70}$ = 0.648; p-value = 0.526) (Table 1). *i.e.,* there were no paddy landraces preferred by the yellow stemborer for ovipositioning. Antixenosis resistance of paddy is expected to influence the oviposition behaviour of the yellow stemborer – selection of landrace for oviposition. In this study, however, antixenosis resistance is possibly not present in all three landraces. Alternatively, a weak level of antixenosis resistance may be present in all three landraces. Unfortunately, under the non-choice test, the resistance is insufficient to deter the female yellow stemborer from oviposition on the paddy plants. A choice test with a larger sample size may further confirm the antixenosis property of these three landraces.

Based on the result, female yellow stemborers clearly preferred to oviposit (based on the average number of egg masses laid) on plant surface (10.14 ± 5.81) instead

Table 1
Mean of egg masses laid by a total of ten females yellow stemborers in each pot planted with paddy plants

Oviposition		Average number of egg masses produced/pot ±SE		
	Landrace	Bajong (n=25)	Bubok (n=23)	Bario (n=25)
Non-plant parts		1.16±0.29	0.91±0.24	1.08±0.35
Plant parts		8.88±1.04	11.00±1.19	10.60±1.28
Stem		1.92±0.41	1.74±0.36	2.32±0.48
Leave	Abaxial	3.92±0.59	5.39±0.96	3.68±0.59
	Adaxial	3.04±0.52	3.87±0.50	4.60±0.56
	Total	6.96±0.92	9.26±1.20	8.28±0.98
Total*		10.04±1.16^{a}	11.91±1.31^{a}	11.68±1.35^{a}

All values were computed by using SPSS software. *Means ±SE within columns followed by the same letter in superscript are not significantly different (One-way ANOVA; P > 0.05)

of on non-plant surfaces (1.06 ± 1.44), regardless of the paddy landraces [two sample t-test; t=12.97; p-value = 0.000]. On plant surfaces, female yellow stemborers were more likely to lay their eggs on the leaves (8.14 ± 5) than stems (2.00 ± 2.06) [two sample t-test; t=9.537; p-value = 0.000]. This is consistent with a study done in Hong Kong, where 94% of the yellow stemborer egg masses were laid on paddy leaves (Thornton et al., 1975). Previous studies also showed that female yellow stemborers preferred to lay their egg masses on leaf blade tip (Karim & Riazuddin, 1999; Pathak & Khan, 1994). The small numbers of egg masses that were oviposited on stem and non-plant surfaces observed in this study may cause by the high saturation of egg masses on the leaf. Previous studies on Lepidopteran indicated that oviposition of females was affected by the presence of egg masses on leaf surfaces. In field observation of Lepidopterans (Nufio & Papaj, 2001; Sielezniew & Stankiewicz-Fiedurek, 2013), adult females tend to avoid ovipositing on a leaf which has egg mass oviposited by females of the same species. Such behaviour is yet to be reported specifically for yellow stemborers, but it is possible that yellow stemborer, a Lepidopteran, may behave similary which resulting in ovipositing on stem and non-plant surface as observed in this study.

In this study, the average numbers of egg masses counted on adaxial was 3.84 ± 2.66 while on abaxial was 4.30 ± 3.59. Between the adaxial and abaxial of a leaf, this study shows an equal likelihood of the female yellow stemborers to oviposit on either side [no significant difference based on two sample t-test; t=-0.892; p-value=0.374]. The result in this study is inconsistent with both Shahjahan (2002) and Thornton et al. (1975), in which the former study showed that the yellow stemborer preferred to oviposit on abaxial surface while the later claimed that the adaxial side of a leaf was a better choice for oviposition by female yellow stemborers. The finding by Shahjahan (2002) is comparable to Renwick and Chew (1994) that explained the abaxial side of a leaf provided protection for the egg masses against predation and parasitism. Moreover, the uneven surface on the abaxial side may provide a suitable footing stage for moths (Renwick & Chew, 1994; Rojas et al., 2018; Teles Pontes et al., 2010). However, no oviposition preference on either the abaxial or adaxial side by female stemborers was observed using a small sample size tested in this study.

CONCLUSION

To summarize, under the non-choice study, there was no significant difference in ovipositional preference of yellow stemborers on the three Sarawak paddy landraces (*i.e.* Bajong, Bario and Bubok). This study provides a preliminary insight to the antixenosis resistance of the three paddy landraces towards the yellow stemborer. The three Sarawak paddy landraces may not be good candidates for breeding a yellow stemborer resistant paddy variety. This study also reveals that the female yellow stemborers preferred to oviposit on the leaves of the paddy instead of the stems. Their ovipositioning preference, however, was not clear between the abaxial or adaxial surface of the leaves.

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REFERENCES

Gumbek, M., & Hamsein, N. N. (2011). Status of paddy pests in Sarawak. In *Proceeding Research Officers Progress Meeting, 4-6 October* (p. 13). Kuching, Malaysia: Department of Agriculture Sarawak.

Hilker, M., & Meiners, T. (2006). Early herbivore alert: Insect eggs induce plant defense. *Journal of Chemical Ecology*, *32*(7), 1379-1397. https://doi.org/10.1007/s10886-006-9057-4

Karim, S., & Riazuddin, S. (1999). Rice insect pests of Pakistan and their control: A lesson from past for sustainable future integrated pest management. *Pakistan Journal of Biological Sciences*, *2*(2), 261–276. https://doi.org/10.3923/pjbs.1999.261.276

Nicholas, D., Hazila, K. K., Chua, H. P., & Rosniyana, A. (2014). Nilai pemakanan dan indeks glisemia varieti beras Bario [Nutritional value and glycemic index of Bario rice varieties]. *Journal of Tropical Agriculture and Food Science*, *42*, 1–8.

Nufio, C. R., & Papaj, D. R. (2001). Host marking behavior in phytophagous insects and parasitoids. *Entomologia Experimentalis et Applicata*, *99*(3), 273–293. https://doi.org/10.1023/A:1019204817341

Pathak, M. D., & Khan, Z. R. (1994). *Insect pests of rice*. Manila, Philippines: International Rice Research Institute. https://doi.org/10.1080/09670878109414180

Renwick, J. A. A., & Chew, F. S. (1994). Oviposition behavior in Lepidoptera. *Annual Review of Entomology*, *39*(1), 377–400.

Rojas, J. C., Kolomiets, M. V., & Bernal, J. S. (2018). Nonsensical choices? Fall armyworm moths choose seemingly best or worst hosts for their larvae, but neonate larvae make their own choices. *PLoS ONE*, *13*(5), 1–29. https://doi.org/10.1371/journal.pone.0197628

Shahjahan, M. D. (2002). Oviposition preference of *Scirpophaga incertulas* (Walker) on different varieties of rice under caged condition. *Pakistan Journal of Scientific and Industrial Research*, *45*(4), 267–272.

Sielezniew, M., & Stankiewicz-Fiedurek, A. M. (2013). Behavioural evidence for a putative oviposition-deterring pheromone in the butterfly, *Phengaris* (*Maculinea*) *teleius* (Lepidoptera: Lycaenidae). *European Journal of Entomology*, *110*(1), 71–80. https://doi.org/10.14411/eje.2013.009

Smith, C. M., & Clement, S. L. (2012). Plant resistance to arthropods: Molecular and conventional approaches. *Annual Review of Entomology*, *57*, 309–328. https://doi.org/10.1007/1-4020-3702-3

Teles Pontes, W. J., Lima, E. R., Cunha, E. G., Teixeira De Andrade, P. M., Lôbo, A. P., & Barros, R. (2010). Physical and chemical cues affect oviposition by *Neoleucinodes elegantalis*. *Physiological Entomology*, *35*(2), 134–139. https://doi.org/10.1111/j.1365-3032.2010.00720.x

Teo, G. K. (2010). *Padi Bajong - A fragrant coloured rice from Lubok Nibong*. Retrieved March 28, 2019, from *https://doa.sarawak.gov.my/modules/web/download_show.php?id=1123*

Thornton, I. W., Marshall, A. T., Kwan, W. H., & Mat, Q. (1975). Studies on Lepidopterous pests of rice crops in Hong Kong, with particular reference to the yellow stem-borer, *Tryporyza incertulas* (Wlk.). *PANS Pest Articles and News Summaries*, *21*(3), 239–251. https://doi.org/10.1080/09670877509411406

Yeo, F. K. S., Kalu, M., Shabdin, Z., Mohamad, N. K., Hussin, N. A., & Chung, H. H. (2018). Diversity of Sarawak rice in Kampung Lebor, Serian – A first insight. In Y. L. Chong, F. K. S. Yeo, & F. A. A. Khan (Eds.), *Glimpses of Bornean biodiversity* (pp. 155–167). Kota Samarahan, Malaysia: UNIMAS Publisher.

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IIUM	*– International Islamic University Malaysia*
JNTBGRI	*– Jawaharlal Nehru Tropical Botanic Garden and Research Institute*
KKP	*– Kementerian Kelautan dan Perikanan (Ministry of Marine Affairs and Fisheries)*
MARDI	*– Malaysian Agricultural Research and Development Institute*
PSU	*– Prince of Songkla University*
TNAU	*– Tamil Nadu Agricultural University*
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UI	*– Universitas Indonesia*
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Revised: June 2019

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MANUSCRIPT PREPARATION

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Pertanika accepts submission of mainly **four** types of manuscripts for peer-review.

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Regular articles are full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide an explanation on research results that contain new and significant findings. Analysis and Discussion must be supported with relevant references.

Size: Generally, these are expected **not exceeding 6000 words** (excluding the abstract, references, tables and/or figures) or between **6 to 12 journal pages,** a maximum of **80 references**, and **an abstract of 100–200 words.**

2. Review article

These report critical evaluation of materials about current research that has already been published by organizing, integrating, and evaluating previously published materials. It summarizes the status of knowledge and outline future directions of research within the journal scope. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged. The manuscript title must start with "Review Article:".

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(b) report/discuss on significant matters of policy and perspective related to the science of the journal, including 'personal' commentary;

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Authors' addresses: Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers.

Tables / figures list: A **list** of number of **black and white / colour figures and tables** should also be indicated on this page. See ***"6. Figures & Photographs"*** for details.

Example (page 2):

In vivo Fecundity Evaluation of Phaleria macrocarpa Extract Supplementation in Male Adult Rats

Sui Sien Leong[1]* and Mohamad Aziz Dollah[2]

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[2]Department of Biomedical Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia

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azizdollah@gmail.com (Mohamad Aziz Dollah), Contact number
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List of Table / Figure:
Table 1.
Figure 1.

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This page should begin with the **Introduction** of your article and followed by the rest of your paper.

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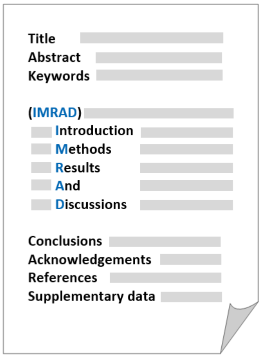

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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words Introduction, Materials and Methods, Results, And, Discussion. It indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: Title, Authors, Keywords, Abstract, Conclusions, and References. Additionally, some papers include Acknowledgments and Appendices.

The Introduction explains the scope and objective of the study in the light of current knowledge on the subject; the Materials and Methods describes how the study was conducted; the Results section reports what was found in the study; and the Discussion section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's instructions to authors.

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Level of heading	Format
1st	**LEFT, BOLD, UPPERCASE**
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  PVY infected Nicotiana tabacum *plants optical density in ELISA*

- Explanatory material should be given in the table legends and footnotes.
- Each table should be prepared on a new page, embedded in the manuscript.
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***** Please submit all tables in Microsoft word format only - because tables submitted as image data cannot be edited for publication and are usually in low-resolution.***

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References begin on their own page and are listed in alphabetical order by the first author's last name. Only references cited within the text should be included. All references should be in 12-point font and double-spaced.

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Books	**Insertion in Text**	**In Reference List**
Book with 1-2 authors	**Information prominent' (the author's name is within parentheses):** … (Cochrane, 2007) … Or **'Author prominent' (the author's name is outside the parentheses):** Cochrane (2007) …	Cochrane, A. (2007). *Understanding urban policy: A critical approach.* Malden, United States: Blackwell Publishing.
Book with 3 or more authors (Pertanika's format)	*For all in-text references, list only the firrst author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Seeley et al., 2011) … Or **'Author prominent' (the author's name is outside the parentheses):** Seeley et al. (2011) …	Seeley, R., VanPutte, C., Regan, J., & Russo, A. (2011). *Seeley's anatomy & physiology*. New York, United States: McGraw-Hill.

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Book with more than 8 authors	*For all in-text references, list only the first author's family name and followed by* 'et al.' A recent study (Edge et al., 2011) concluded that… Or Edge et al. (2011) concluded that ….	For books with eight or more authors, please follow the guidelines for journal articles with eight or more authors.
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Editor	**Information prominent' (the author's name is within parentheses):** … (Zairi, 1999) … Or **'Author prominent' (the author's name is outside the parentheses):** Zairi (1999) …	Zairi, M. (Ed.). (1999). *Best practice: Process innovation management.* Oxford, United Kingdom: Butterworth- Heinemann.

Books	Insertion in Text	In Reference List
Several works by same author in the same year	**Information prominent' (the author's name is within parentheses):** … (Fullan, 1996a, 1997b) … Or **'Author prominent' (the author's name is outside the parentheses):** Fullan (1996a, 1996b) …	Fullan, M. (1996a). Leadership for change. In *International handbook for educational leadership and administration.* New York, United States: Kluwer Academic. Fullan, M. (1996b). *The new meaning of educational change.* London, United Kingdom: Casell.
Several authors, different years referred to collectively in your work	**List sources alphabetically by family name in the in-text reference in the order in which they appear in the Reference List.** The cyclical process (Carr & Kemmis, 1986; Dick, 2000) suggests…	Carr, W., & Kemmis, S. (1986). *Becoming critical: Education knowledge and action research.* London, United Kingdom: Falmer Press. Dick, B. (2000). *A beginner's guide to action research.* Retrieved June 1, 2019, from http://www.scu.edu.au/schools/gcm/ar/arp/guide.html

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Journal article with 1-2 authors	**Information prominent' (the author's name is within parentheses):** … (Kramer & Bloggs, 2002)… Or **'Author prominent' (the author's name is outside the parentheses):** Kramer and Bloggs (2002) …	Kramer, E., & Bloggs, T. (2002). On quality in art and art therapy. *American Journal of Art Therapy*, *40*, 218-231.
Journal article with 3 or more authors (Pertanika's format)	*For all in-text references, list only the firrst author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Erlo et al., 2008) … Or **'Author prominent' (the author's name is outside the parentheses):** Erlo et al. (2008) …	Elo, A., Ervasti, J., Kuosma, E., & Mattila, P. (2008). Evaluation of an organizational stress management program in a municipal public works organization. *Journal of Occupational Health Psychology*, *13*(1), 10-23. doi: 10.1037/1076-8998.13.1.10

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Journal article with more than 8 or more authors	**Information prominent' (the author's name is within parentheses):** … (Steel et al., 2010).. Or **'Author prominent' (the author's name is outside the parentheses):** Steel et al. (2010) …	Steel, J., Youssef, M., Pfeifer, R., Ramirez, J. M., Probst, C., Sellei, R., ... Pape, H. C. (2010). Health-related quality of life in patients with multiple injuries and traumatic brain injury 10+ years postinjury. *Journal of Trauma: Injury, Infection, and Critical Care, 69*(3), 523-531. doi: 10.1097/TA.0b013e3181e90c24
Journal article with DOI	**Information prominent' (the author's name is within parentheses):** … (Shaw et al., 2005).. Or **'Author prominent' (the author's name is outside the parentheses):** Shaw et al. (2005) …	Shaw, K., O'Rourke, P., Del Mar, C., & Kenardy, J. (2005). Psychological interventions for overweight or obesity. *The Cochrane database of systematic reviews* (2). doi:10.1002/14651858.CD003818.pub2

Newspapers

Newspapers	**Insertion in Text**	**In Reference List**
Newspaper article – with an author	… (Waterford, 2007)...	Waterford, J. (2007, May 30). Bill of rights gets it wrong. *The Canberra Times*, p. 11.
Newspaper article – without an author	… ("Internet pioneer", 2007) …	Internet pioneer to oversee network redesign. (2007, May 28). *The Canberra Times*, p. 15.
Article in an newsletter	... ("Australians and the Western Front", 2009) ...	Australians and the Western Front. (2009, November). *Ozculture newsletter*. Retrieved June 1, 2019, from http://www.cultureandrecreation.gov.au/ newsletter/

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Conference / Seminar Papers	Insertion in Text	In Reference List
Print – *If the paper is from a book, use the book chapter citation format. If it is from regularly published proceedings (e.g. annual), use the Journal article citation format.*	… (Edge, 1996) … Or Edge (1996) …	Edge, M. (1996). Lifetime prediction: Fact or fancy? In M. S. Koch, T. Padfield, J. S. Johnsen, & U. B. Kejser (Eds.), *Proceedings of the Conference on Research Techniques in Photographic Conservation* (pp. 97-100). Copenhagen, Denmark: Royal Danish Academy of Fine Arts.
Online	… (Tester, 2008) … Or Tester (2008) …	Tester, J. W. (2008). The future of geothermal energy as a major global energy supplier. In H. Gurgenci & A. R. Budd (Eds.), *Proceedings of the Sir Mark Oliphant International Frontiers of Science and Technology Australian Geothermal Energy Conference*, Canberra, Australia: Geoscience Australia. Retrieved June 1, 2019, from http://www.ga.gov.au/image_cache/ GA11825.pdf

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Government as author	**First in-text reference: Spell out the full name with the abbreviation of the body.** … (Department of Finance and Administration [DOFA], 2006) … **Subsequent in-text reference/s:** **Use the abbreviation of the body.** … (DOFA, 2006)…	Department of Finance and Administration. (2006). Delivering Australian Government services: Managing multiple channels. Canberra, Australia: Author.
Government report - online	**First in-text reference: Spell out the full name with the abbreviation of the body.** … (Department of the Prime Minister and Cabinet [PM&C], 2008) … **Subsequent in-text reference/s:** **Use the abbreviation of the body.** … (PM&C, 2008)…	Department of the Prime Minister and Cabinet. (2008). *Families in Australia: 2008.* Retrieved June 1, 2019, from http://www.....

8. General Guidelines

Abbreviations: Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

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